Insights into the role of almond CBF transcription factors in the environmental control of cold acclimation and dormancy break

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Aos meus pais

"(…) coisa simples
e pouca, mas destino:
crescer como árvore, resistir
ao vento, ao rigor da invernia,
e certa manhã sentir os passos
de Abril
ou, quem sabe?, a floração
dos ramos, que pareciam
secos, e de novo estremecem
com o repentino canto da cotovia."

Eugénio de Andrade

(in O lugar da Casa)

Almonds Are In!

Proceedings of the 26th Annual
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LIST OF ABBREVIATIONS

aa	amino acid
ABA	Abcisic Acid
ATP	Adenosine triphosphate
bp	base pair
BLAST	Basic Local Alignment Search Tool
BC	Before Christ
BSA	Bovine Serum Albumin
CBF	C-repeat Binding Factor
CAMV35S	Cauliflower mosaic virus 35S promoter
cM	centimorgan
CRs	Chilling Requirements
COR	Cold responsive
cDNA	complementary DNA
cpm	counts per minute
DAG	Days After Germination
ºC	degrees Celsius
DHN	Dehydrin
dNTPs	Deoxynucleotide Triphosphates
DNA	Deoxyribonucleic acid
DAM	DORMANCY ASSOCIATED MADS-box
EDTA	Ethylene Diamine Tetraacetic Acid
EVG	EVERGROWING
FAO	Food Agriculture Organization
GA	Gibberelic Acid
g	grams
HRs	Heat Requirements
HA	Hemagglutinin
h	hours
iPCR	inverse PCR
kDa	kilodalton
Lat.	Latitude
LG	Linkage Group
L	Litre
LD	Long Day
Long.	Longitude
LUC	Luciferase
Mbp	Megabase pair
µg	microgram
µL	microlitre
µM	micromolar
mg	milligram

mL	millilitre
mM	millimolar
Mt	Million tones
m35S	minimal 35S promoter
min	minutes
M	Molar
ng	nanograms
nm	nanometer
PCR	Polymerase Chain Reaction
Prd	*Prunus dulcis*
Pp	*Prunus persica*
QTLs	Quantitative Trait Loci
RACE	Rapid Amplification of cDNA ends
RT-PCR	Reverse Transcription - PCR
RNA	Ribonucleic acid
RdDM	RNA-directed DNA methylation
RT	Room Temperature
sec	seconds
SD	Short Day
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide gel electrophoresis
TxE	Texas x Earlygold
TFs	Transcription Factors
TEs	Transposable Elements
U	Units
UTR	Untranslated Region
v	volume
w	weight
GUS	β-glucuronidase

SUMMARY

Cold is an important environmental factor that may influence plant survival and productivity. It also plays a crucial role in the seasonal development of perennial plants by regulating the extent of (winter) dormancy and that of vegetative and reproductive growth periods. This aspect is particularly relevant in fruit trees, since it may directly impact fruit production. The *Prunus* genus includes economically relevant species, and the almond (*Prunus dulcis* Mill.) stands out for its high range of genetic variability, thus being an interesting model to study specific traits of agricultural interest. Considering that low temperature signalling pathways are still poorly understood in *Prunus* spp., this study aimed to identify and elucidate, in almonds, the function of members of the C-repeat Binding Factor (CBF) family of transcription factors (TFs) which are known to be relevant in the response to cold. Therefore, two *CBF* genes (*PrdCBF1* and -*2*) and their corresponding regulatory regions were cloned and sequenced in this species. Southern-blot analysis and further comparisons to the recently available peach genome revealed that the almond *CBF* family is composed of at least five members, located in close proximity in chromosome 5. In addition, a mapping analysis suggested an association between *PrdCBF*s and *PrdDehydrin1* (*PrdDHN1*, a putative CBF target gene, involved in cold acclimation) to recently reported QTLs controlling chilling requirements and blooming time. However, further analyses are necessary to confirm this association.

During cold treatments applied to *in vitro* almond plantlets, *PrdCBF1* and *PrdCBF2* transcription was rapidly induced, modulated either by light and/or the circadian rhythm. Similar modulations were observed under control conditions in the expression of *PrdDHN1*, although cold stress induced its constitutive expression, thus demonstrating its role in cold acclimation. The role of PrdCBF1 and PrdCBF2 as functional TFs was

confirmed by heterologous expression in *Arabidopsis*. Both genes proved to be functional during transient expression *in vitro*, activating the expression of a reporter gene under the control of promoter sequences containing CBF-specific recognition sites. *PrdCBF2* also proved to be functional when constitutively overexpressed *in vivo*, inducing endogenous CBF-target genes under control conditions.

Considering the role of temperature perception in fall/winter dormancy and cold acclimation, we followed the expression *PrdCBF1*, *PrdCBF2* and *PrdDHN1* genes in flower buds and shoots from adult almond trees throughout the natural dormancy period and until growth resumption. Candidate genes related to floral development were also studied in flower buds. In two consecutive years, *PrdCBF gene*s showed somewhat distinct patterns of expression during dormancy stages, although *PrdCBF2* transcript accumulation suggested a closer correlation to cold acclimation. Interestingly, transcript levels of both *PrdCBF*s and *PrdDHN1* decreased in mid-winter stages, in close correlation to ecodormancy break and blooming. These results were obtained in both shoots and flower buds, associating the expression of these genes to a global deacclimation mechanism expected during growth resumption. Flower bud development during ecodormancy break was also observed by a gradual increase in the transcript levels of two homeotic genes related to floral organ development (*PrdMADS1* and -*3*). In addition, the differential expression of two genes related to gibberellin biosynthesis and catabolism (*PrdGA20OX* and *PrdGA2OX*, respectively) supported the role of gibberellin metabolism in ecodormancy break. Finally, in a preliminary study of the epigenetic regulation of dormancy, we determined the DNA methylation pattern of a specific promoter region from *PrdCBF2* prior and following flower bud break. Although no difference was observed between the stages, a specific region of the analysed sequence was found to be a methylation target site. The occurrence of methylated

cytosines within this site might be involved in the regulation of *PrdCBF2* gene expression in specific floral organs.

In conclusion, this work provides the first evidence for the role of *CBF* genes during seasonal cold acclimation and ecodormancy break in a fruit tree species. In addition, new genetic markers for dormancy-activity transitions in flower buds are also proposed.

SUMÁRIO

O frio é um importante fator ambiental que pode influenciar a sobrevivência e produtividade das plantas, desempenhando também um papel crucial no desenvolvimento sazonal de plantas perenes ao regular a duração da dormência (no inverno) e dos períodos de crescimento vegetativo e reprodutivo. Este aspeto é particularmente importante em árvores fruteiras, uma vez que pode ter um impacto direto na produção dos frutos. O género *Prunus* inclui espécies de elevado interesse económico, e a amendoeira (*P. dulcis* Mill.) destaca-se pela sua elevada variabilidade genética, sendo por isso um modelo interessante para o estudo de características específicas com interesse agronómico. Considerando que as vias de sinalização de baixas temperaturas são ainda pouco compreendidas em *Prunus* spp., este estudo pretendeu identificar e elucidar, em amendoeira, a função de membros da família de fatores de transcrição "C-repeat Binding Factor" (CBF), conhecidos pelo seu papel relevante na resposta ao frio. Assim, foram clonados e sequenciados nesta espécie dois genes *CBF* (*PrdCBF1* e *-2*) e respetivas regiões reguladoras. Análise por "Southern blot" e comparações adicionais no genoma de pessegueiro recentemente disponibilizado, revelou que a família de CBFs em amendoeira é composta de pelo menos cinco membros, localizados em proximidade no cromossoma 5. Adicionalmente, a análise de mapeamento sugeriu uma associação dos *PrdCBF*s e de *PrdDehydrin1* (*PrdDHN1*, um

provável gene alvo dos CBFs, envolvido na aclimatação ao frio) a "QTLs" recentemente referidos como envolvidos no controlo dos requesitos de frio e período de floração. Para confirmar esta associação serão, no entanto, necessárias análises adicionais.

Durante os tratamentos de frio aplicados a plântulas de amendoeira *in vitro*, a transcrição de *PrdCBF1* e *PrdCBF2* foi rapidamente induzida, sendo modulada pela luz e/ou ritmo circadiano. Semelhante modulação foi observada na expressão de *PrdDHN1* em condições controlo, embora o stress de frio tenha induzido a sua expressão constitutiva demonstrando assim o seu papel na aclimatação ao frio. O papel de PrdCBF1 e PrdCBF2 como fatores de transcrição funcionais foi confirmado por expressão heteróloga em *Arabidopsis*. Ambos os genes demonstraram ser funcionais durante a sua expressão transiente em protoplastos, ativando a expressão de um gene repórter sob o controlo de sequências promotoras contendo regiões de reconhecimento específicas de CBFs. *PrdCBF2* também provou ser funcional por sobre-expressão constitutiva *in planta*, levando à indução em condições controlo de genes endógenos conhecidos como genes alvo de CBFs.

Considerando o papel da perceção da temperatura na dormência e aclimatação ao frio de outono/inverno, seguimos a expressão dos genes *PrdCBF1*, *PrdCBF2* e *PrdDHN1* em botões florais e ramos de amendoeiras adultas, durante o período natural de dormência até ao recomeço do crescimento. Genes candidatos relacionados com o desenvolvimento floral foram também estudados em botões florais. Em dois anos consecutivos, os dois genes *PrdCBF* apresentaram padrões de expressão um pouco distintos durante os estádios de dormência, embora a acumulação de transcritos de *PrdCBF2* tenha sugerido uma correlação mais próxima com a aclimatação ao frio. Interessantemente, os níveis de transcrito dos dois genes *PrdCBF* e de *PrdDHN1* diminuíram nos estádios de inverno, numa correlação próxima à quebra da ecodormência e floração. Estes resultados

foram obtidos nos botões florais e nos ramos, associando a expressão destes genes a um mecanismo de desaclimatação global expectável durante a reativação do crescimento. O desenvolvimento nos botões florais durante a ecodormência foi também acompanhado por um aumento gradual nos níveis de transcritos de dois genes homeóticos relacionados com o desenvolvimento dos órgãos florais (*PrdMADS1* e *PrdMADS3*). Adicionalmente, a expressão diferencial de dois genes relacionados com a biossíntese e catabolismo das giberelinas (*PrdGA20OX* e *PrdGA2OX*, respetivamente) suporta o papel do metabolismo desta hormona na quebra da ecodormência. Por fim, num estudo preliminar sobre a regulação epigenética da dormência, determinou-se o padrão de metilação do DNA numa região específica do promotor de *PrdCBF2*, antes e depois da floração. Embora não tenham sido observadas diferenças entre os dois estádios, um provável local alvo de metilação foi encontrado numa região específica da sequência analisada. A presença de citosinas metiladas nesta região poderá estar relacionada com a regulação da expressão do gene *PrdCBF2* em órgãos florais específicos.

Em conclusão, este trabalho fornece pela primeira vez numa árvore fruteira indícios sobre o envolvimento de genes CBF durante a aclimatação ao frio e a quebra da ecodormência sazonal. Adicionalmente, são também propostos novos marcadores genéticos para as transições entre dormência e atividade em botões florais.

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CHAPTER I

General Introduction

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1. Seasonal development and its impact in fruit trees productivity

In boreal and temperate zones, continuous plant growth and development is incompatible with the perennial habit, given the chilling and freezing conditions observed during winter. The long life span, over many seasons, attributed to these plants is only possible due to an adaptation that allows temporary growth cessation in active tissues, combined with an enhanced ability to cope with such harsh winter conditions. This seasonal dormancy regulates the timing and duration of the following growing season, generally starting close or during spring. Increasing research has been focusing on the study of the signalling mechanisms involved in this response in perennial tree models. Some connections with the well-known flowering induction pathways from annuals herbaceous models have been identified, as well as other perennial-specific adaptations (Horvath, 2009; van der Schoot and Rinne, 2011).

Studies in woody models have been made, but mostly in vegetative buds or during juvenile phases. However, in fruit trees, and particularly within the Rosaceae family, fall/winter dormancy affects not only vegetative buds, but also flowering buds developing since spring or summer. Blooming time in the following growing season also appears to be regulated by the control of the dormancy state. Thus, understanding this process in these plants is very important to control the timing of fruit development and yield. In this chapter, the emerging concepts related to seasonal dormancy in woody plants will be reviewed in the light of the current topics associated with crop improvement in Rosaceae fruit trees.

2. Rosaceae fruit trees

The Rosaceae family has a high economical value, being the third most important plant family in temperate regions (Dirlewanger et al., 2002), after the Poaceae (grasses) and Fabaceae (legumes) families. It includes

some of the most widely produced edible fruit species in the world, such as pome fruits from Maloideae (e.g. apple, pear and quince), stone fruits from Prunoideae (e.g. peach, apricot, cherry, plum, almond) and berries from Rosoideae (e.g. strawberry and raspberry), as well as important ornamental and timber species. The current importance of edible rosaceous crops resulted from centuries of selection and domestication, which generated a dramatic enrichment of fleshy fruit or nut size and flavours. Although no member of this family is considered a staple food, its edible fruits or nuts are in high demand for their nutritional values (Shulaev et al., 2008). They provide unique contributions to dietary options of consumers and overall human health, since they are natural sources of important nutrients and phytochemicals (antioxidants and phenolic compounds) (Shulaev et al., 2008). According to the Food Agriculture Organization (FAO, http://faostat.fao.org), from 2000 to 2009, the average world production of edible rosaceous fruits was close to 121 million tones (Mt)[1*]. An increasing tendency was observed during this period, and in 2009 total production reached 140 Mt[1]. The fruits mainly contributing for this achievement are apple (*Malus* spp.), pear (*Pyrus* spp.) and several *Prunus* species, accounting for approximately 52%, 16% and 29% of global production, respectively (Figure 1a).

The fruits derived from *Prunus* spp. are often referred as drupes or stone fruits, since their seeds are enclosed within a hard-lignified endocarp. Within *Prunus*, the most economically relevant species, in terms of global production, is peach (*Prunus persica* [L.] Batsch) (Figure 1a), also showing the clearest increase in production over the last decade[1]. Other high producing species are fleshy fruit-producing trees such as plum (e.g. *Prunus domestica* L.) and apricot (*Prunus armeniaca* L.). Although not as relevant,

[1] According to FAO world statistics of crop production. Average values were calculated for 10 years (2000 to 2009), http://faostat.fao.org.

[*] Not considering edible Rosaceae fruits (e.g. loquat, rose hips) included in heterogeneous groups of species belonging to different families, with minor relevance at the international level.

almond (*Prunus dulcis* [Miller] D.A. Webb) stands out as one of the most important nut producing trees (Figure 1b). Almond production also increased over the last decade, with an average production of almost 2 Mt[1] (19% of global nut tree production). The United States of America stands as the major almond producer, holding close to 45% of world's production during the last decade, followed by Spain, Iran, Italy, Morocco and Syria. Other European countries such as Greece, Portugal and France are also relevant producers, although in the last two a demarked drop in production was observed until 2009[1].

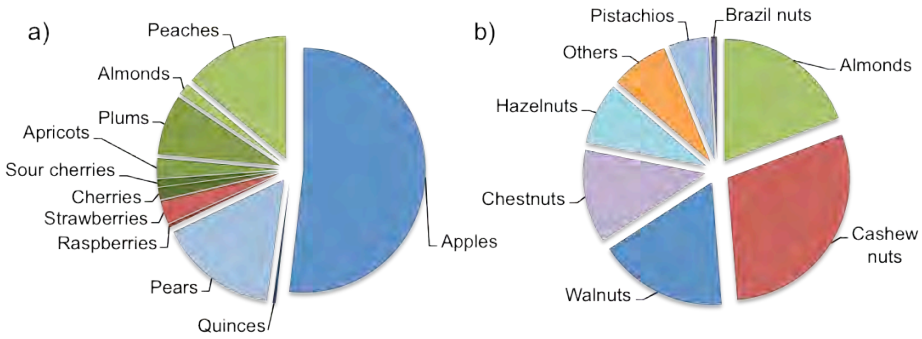

Figure 1. Average percentage of annual production of fruit species from Rosaceae (a) and nut tree species (b). Calculations were made based on production values from 2000 to 2009 reported by FAO. In pie chart (a) pome fruits are represented in blue colours, stone fruits in green and berries in red.

The profitable production of Rosaceae fruits and nuts can be challenging given the long generation cycles, plant size and complex reproductive biology for breeding approaches. In addition, the necessary balance of quality expectations with yield requirements, cost efficiency and shipping/marketing constraints at the pos-harvest level also need to be considered (Shulaev et al., 2008). In order to improve traditional breeding, important traits such as fruit size, shape and flavour, yield and plant response to either biotic or abiotic stress are being targeted at the agronomical, genetic and molecular research levels.

3. Almond – a model plant for genetic studies in fruit trees

Prunus genus include a large group of deciduous or evergreen trees and shrubs, divided in five subgenera, although the main cultivated species are only distributed among three of these: *Prunophora* (plum and apricot), *Amygdalus* (almond and peach) and *Cerasus* (sour and sweet cherries) (Westwood, 1993; Badenes and Parfitt, 1995). It is hypothesized that *Cerasus* diverged earlier from the ancestral *Prunus* species, being genetically more distant from *Prunophora* and *Amygdalus* (Badenes and Parfitt, 1995).

Several *Prunus* species such as peach, almond, apricot or sweet cherry are characterized by a small genome size and low ploidy level (2*n*=16), being good candidates for a woody species plant model (Oliveira et al., 2008). Due to its higher economic relevance, shorter juvenile period (2-3 years) and self-compatibility for breeding, peach is currently the most well characterized species for this genus (Shulaev et al., 2008; Fan et al., 2010). A draft assembled and annotated peach genome is now available (http://www.rosaceae.org/peach/genome, International Peach Genome Initiative, 2010). The currently estimated peach genome size ranges from 230 to 240 Mbp, close to the double of that of the herbaceous plant model *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000).

Despite the diversity in both form and function, species belonging to the *Prunus* genus display a high degree of genome conservation, and crosses between closely related species, particularly from the same subgenus, are possible and may produce fertile hybrids (Shulaev et al., 2008). Besides the development of new fruit varieties (Hummer and Janick, 2009) the ability to perform interspecific crosses also allowed the development of the almond (cv. ‘Texas’) x peach (cv. ‘Earlygold’) genetic map (TxE), which is currently the reference map for *Prunus*. This map has been a useful tool for the research in this genus, since it provided a highly

polymorphic population for linkage studies, establishing a common terminology for linkage groups (LGs). It also provided a set of transferable markers of known position, which facilitated the development of framework maps in other crosses (Dirlewanger et al., 2004). In this way, when comparing the positions of anchor (transferable) markers of the TxE map with those of several other maps from other *Prunus* populations, it was observed that the genomes of the diploid species are almost collinear. Therefore, it was proposed that this genus can be treated as a single genetic entity (Dirlewanger et al., 2004) and other closely related *Prunus* species, such as almond, may be used as a source of genetic variability for the study of specific traits.

The origin of cultivated almond relates to one or more of the many wild species that evolved in the deserts and lower mountain slopes of Central and Southwest Asia, presently China and Iran (Oliveira et al., 2008). Although there are several hypotheses related to specific almond ancestors and domestication events, it is assumed that mankind played an important role in breeding and propagation of the current cultivated species and respective varieties. Almond introduction in the Mediterranean regions, occurred through the Phoenicians, and around 450 BC almond culture spread from Greece to all Mediterranean coast, with several important centres appearing in the Iberian Peninsula, France, Italy, Morocco, Tunisia and Turkey (Kester and Ross, 1996). Nowadays, almond growth cycle is adapted to a Mediterranean type climate, being found in many regions near the Mediterranean coast but also in other countries like the USA (mostly California) or Australia.

Cultivated almond is an average size deciduous tree (5 to 12 meters) with a strong root system, narrow, long and sharp-pointed leaves, and white to pink flowers. Almond culture requires a hot and dry climate during summer, resisting well to drought when the rootstock is appropriate (Oliveira et al., 2008). After a period of juvenile vegetative growth, which in

almond ranges from 3-6 years (Monteiro et al., 2003), plants achieve reproductive competence and enter the adult stage. As in other *Prunus* species growing in the Northern hemisphere, almond flower induction occurs during summer (Oliveira et al., 2008).

Almond, as opposed to other *Prunus* spp. like peach, is predominantly self-incompatible, a trait that promotes outcrossing during breeding events thus maintaining the hybrid vigour (Oliveira et al., 2008). This self-incompatibility is gametophytic and it is controlled by a single locus with multiple codominant alleles (Socias i Company and Felipe 1988; Dicenta and García 1993). Although being a limitation in controlled breeding programs, this trait had a major impact during evolution, as almond is currently among the most polymorphic of all cultivated fruit and nut species (Martinez-Gomez et al., 2007). This genetic variability, combined with a series of molecular tools (e.g. *in vitro* propagation, genetic transformation) and genetic data already developed for this species (Martinez-Gomez et al., 2007; Oliveira et al., 2008; Arús et al., 2009) could be used to study specific traits, which would later support breeding projects in *Prunus* or other Rosaceae.

4. Blooming time and chilling requirements in fruit production

Developing flowers and vegetative meristems from most Rosaceae fruit trees are able to arrest growth in fall, as an avoidance mechanism for the harsh winter conditions. During winter dormancy, these tissues remain enclosed within protective scale leaves surrounding the buds. Growth reactivation and anthesis occurs only in the next year, from late-winter to spring (Tabuenca et al., 1972; Egea et al., 2003; J. M. Alonso et al., 2005; Ruiz et al., 2007). In *Prunus*, as in most fruit species, blooming time is considered as a quantitative and highly heritable trait (Campoy et al., 2011). In fact, several quantitative trait loci (QTLs) associated with blooming time

have been reported in almond and peach intra and interspecific populations (Joobeur et al., 1998; Dirlewanger et al., 1999; Sánchez-Pérez et al., 2007; Fan et al., 2010). Some of them showed to co-localize among different maps, as for example in the central part of LG4 and LG7. Additionally, a late blooming allele (*Lb*) was also described for almond, which is also located in the central part of LG4 and appeared to be dominant over early flowering, conferring an average delay of 15 days in bloom (Ballester et al., 2003).

Blooming time is dependent on winter weather conditions and related to temperature requirements, which vary among species and genotypes (e.g. Tabuenca, et al. 1972; Egea et al., 2003; Alonso et al., 2005; Ruiz et al., 2007). Chilling requirements (CRs) for dormancy break prevent trees from initiating growth in response to transient warm-promotive temperatures occurring early in winter, being in most cases, the major factor determining blooming date in *Prunus* (Egea et al., 2003; Ruiz et al., 2007; Alburquerque et al., 2008). This trait is assumed as a result of long-term adaptation of genotypes developed in different climatic regions. Many mathematical models have been proposed to evaluate genotype-specific CRs, calculating either the number of hours at which air temperature is in a certain range (assuming that all temperatures in this range are equally effective) or the number of chilling units, determined by attributing different weight factors to temperatures in different ranges (Fan et al., 2010; Campoy et al., 2011). However, due to the lack of knowledge related to the genetic and physiological mechanisms regulating CRs, these models have been used only to fit the blooming responses of tree species to local weather conditions (Fan et al., 2010).

In apricot vegetative buds, a major gene with dominance for low CRs was suggested (Tzonev and Erez, 2003). Recently, several QTLs associated with CRs have been found for peach and apricot (Fan et al., 2010; Olukolu et al., 2009). In peach, these QTLs were distributed among six of the defined LGs (excluding LG2 and -3), and two of them, which

mapped onto LG1 and LG7, were considered major QTLs explaining approximately 40% and 18% of the phenotypic variation, respectively (Fan et al., 2010). The extensive co-localization of QTLs for CRs and blooming time observed in this particular study (and also observed in some extent when comparing to mapped QTLs on the TxE reference map) suggested the occurrence of a unified temperature sensing and action system regulating these traits (Fan et al., 2010). This observation could be reinforced by the results previously obtained for apricot (Olukolu et al., 2009), in which several QTLs for CRs were localized in similar map positions to those obtained for peach (Fan et al., 2010).

While CRs negatively regulate dormancy, heat requirements (HRs) may control the promotive signals leading to blooming or vegetative bud break. However, this aspect has been less studied and there is still little evidence regarding the timing at which HRs start to be perceived (prior to or after CR fulfilment). Common methods to determine HRs consist in calculating heat accumulation above a threshold, from the CR fulfilment to anthesis (Campoy et al., 2011). However, while blooming time and CR appear to be genetically determined, it is still not clear if the same occurs for heat requirements (Fan et al., 2010). In fact, CRs may control HRs to some extent, considering that extended exposure to chill temperatures (exceeding CRs) may result in the variation of HRs. According to a recently proposed model, there may be a critical and optimum CR and, between these parameters, a period of interaction between chilling and warm temperatures that in many combinations may lead to bud break (Harrington et al., 2010).

Within Rosaceae, *Prunus* spp. tend to flower earlier, making them very susceptible to spring frosts, thus requiring the selection of genotypes more adjusted to a specific environment. In addition to a direct impact on flower integrity, early blooming may impact fruit production also due to the lack of pollinators, like honeybees, for an efficient cross-pollination after anthesis. Nevertheless, while late blooming is generally a target in breeding

programs, genotypes with high CRs may suffer inadequate chilling in warm years (or regions) leading to irregular bud break and, consequently, poor fruit set (Topp et al., 2008). On the other hand, early ripening cultivars (associated with early blooming and low CR) may be preferred in particular environmental or culture contexts, since early ripening fruits have often higher commercial value (Ruiz et al., 2007; Topp et al., 2008).

Given that the goals established in the culture of fruit trees may follow different trends, a more detailed knowledge of the mechanisms controlling dormancy break in flower buds may be important to establish profitable and competitive orchards.

5. Molecular mechanisms controlling dormancy in woody plants

Plant growth and development, in the context of a sessile existence, allowed the development of elaborated mechanisms for surviving under unfavourable growing conditions experienced in nature. Plant adaptation and survival in the perennial habit requires developmental transitions between active growth and dormant stages, in straight synchrony with surrounding environment. Therefore, perenniality requires at least one indeterminate meristem that continues vegetative growth on the following season (Rohde and Bhalerao, 2007). The first most widely known definition of dormancy relates to ‘absence of visible growth in any plant structure containing a meristem’ (Lang, 1987). According to Lang et al. (1987) fall/winter dormancy may be divided in two different stages (Figure 2). In early autumn, buds of most perennial plants become endodormant as a consequence of reducing day length and temperatures. During this stage, repression of growth and development persists even under environmental conditions that in a different context would favour growth. Endodormancy is maintained by endogenous factors in a straight correlation with specific chilling requirements, which later contribute to restore growth ability.

However, growth reactivation is only possible after the onset of growth-promotive conditions, which often relate to warmer temperatures in spring. Therefore, this quiescent stage following endodormancy is referred as ecodormancy and is maintained under low temperatures and released by HRs. In addition, the concept of paradormancy was also developed, corresponding to the growth inhibition arising from another part of the plant, as observed in apical dominance during active growing seasons (Figure 2) (Lang et al., 1987; Horvath, 2009).

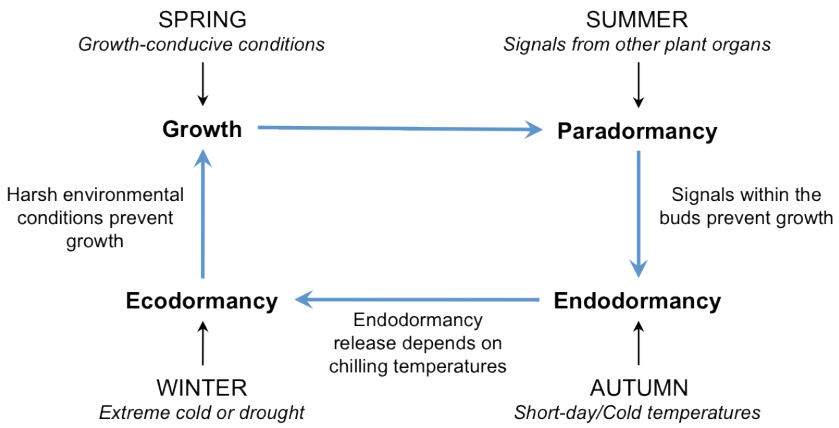

Figure 2. Schematic representation of common seasonal transitions of dormancy and growth in the perennial habit. Adapted from Horvath (2009).

Considering that meristem growth is not readily visible and absence of growth is in fact related to an inability to resume growth, the previous description of dormancy is only appropriate in a broader level (the complete bud), not considering the cellular mechanisms governing activity-dormancy transitions in different tissues (Rohde and Bhalerao, 2007). Rohde and Bhalerao (2007) proposed that dormancy is the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favourable conditions. Although more detailed, this definition does not consider the period related to ecodormancy, which is still accounted in studies regarding perennial plants.

In more detail, annual cycling in perennial plants involves growth cessation, bud set, dormancy (induction, maintenance and release) and cold hardiness development (cold acclimation), which are sequential, but still, interconnected processes (Tanino et al., 2010). Cold acclimation enables plant survival under freezing conditions and appears to be dependent on growth cessation (Weiser, 1970; Ruttink et al., 2007; Kalcsits et al., 2009) rather than dormancy induction. In turn, dormancy appears to be important in the maintenance and release of cold hardiness, but not in its initiation (Tanino et al., 2010).

5.1. Dormancy induction

Activity-dormancy transitions in woody plants involve extensive morphological and physiological changes in meristems and surrounding tissues, which prevent cell growth and proliferation. In vegetative buds, these changes include arrest of leaf primordial initiation and formation of bud scales enclosing the former and the shoot apical meristem (Rohde et al., 2002). Simultaneously, plasmodesmatal connections are closed through net callose deposition, leading to symplastic isolation of the meristems (Rinne et al., 2001). This fact may inhibit leaf primordial development, as nutrient import is crucial to sustain morphogenesis. In addition, dormancy induction also appears to cause a decrease in water content and osmotic potential in those organs (Welling et al., 1997; Rinne et al., 1998; Welling et al., 2002, 2004). This decrease may be related to the impregnation of cell walls with yet unidentified substances that impede movement of water, as well as water-soluble ligands and other molecules (van der Schoot and Rinne, 2011).

At the molecular level, the transition between active to dormant states also involves an extensive reprogramming of transcription and metabolism (Schrader et al., 2004; Bassett et al., 2006; Druart et al., 2007; Ruttink et al., 2007; Karlberg et al., 2010). During early dormancy stages, a

shift in metabolism leads to the accumulation of storage compounds such as sucrose, fatty acids or products resulting from starch breakdown, which support the production of cryoprotectants and energy generation (Schrader et al., 2004; Druart et al., 2007). Considering that sufficient reserves need to be stored in overwintering parts to allow cold acclimation, a timely cessation may be an important part of the survival strategy in perennials (van der Schoot and Rinne, 2011).

It is well known that short day (SD) perception is able to trigger growth cessation and development of dormancy in woody trees (Wareing, 1956). Plants measure day length by measuring duration of the night, and if it exceeds the genetically determined 'critical day length' it is sensed as SD (Welling and Palva, 2006). The critical photoperiod marks the transition between active growth to cessation of growth (stopping of internode elongation) and bud set. It was previously suggested that phytochrome A (PhyA) plays an important role in SD perception. Overexpression of an oat PhyA gene in hybrid aspen (*Populus tremula x tremuloides*) generated plants less sensitive to the SD signal, consequently preventing SD-induced growth cessation and cold acclimation (Olsen et al., 1997; Welling et al., 2002). In turn, transgenic lines showing reduced levels of the endogenous PhyA responded faster to daylength shortening, inducing earlier growth cessation and bud set as opposed to wild type (Kozarewa et al., 2010). It was also observed that clock regulated periods are lengthened as levels of PhyA decrease, due to its effect on the expression of *LATE ELONGATED HYPOCOTYL* (*LHY*), an important circadian oscillator gene (Kozarewa et al., 2010). Thus, the level of PhyA strongly influences seasonally regulated growth in *Populus*, being a central coordinator between internal clock-regulated rhythms and external light/dark cycles (Kozarewa et al., 2010).

Similar to *Arabidopsis*, the perception of a critical photoperiod in *Populus* trees results in a clock-regulated change in the output of the CONSTANS (CO)/FLOWERING LOCUS T (FT) module (Böhlenius et al.,

2006). In *Arabidopsis*, *CO* is a circadian-clock regulated gene, in part regulated by PhyA (Yanovsky and Kay, 2002), which displays a diurnal regulation. Its transcripts accumulate at the end of day in long days (LD) and during the night in SD (Suárez-López et al., 2001). This gene encodes a transcription factor responsible for the induction of *FT*, a signalling element involved in the activation of floral meristem identity genes (Wigge et al., 2005). Since CO is dark-labile, significant FT signal is only produced towards LDs (Valverde et al., 2004), inducing flowering in this context. In *Populus*, overexpression of two FT orthologs (*FT1-2*), besides inducing early flowering (Hsu et al., 2006) also inhibited SD-induced growth cessation (Böhlenius et al., 2006). In agreement, endogenous *FT* levels, showed to be downregulated in wild-type plants grown under SD, while under LD they showed a circadian-like expression similar to *Arabidopsis* (Böhlenius et al., 2006). Downregulation of *CENTRORADIALIS-LIKE1* (*CENL1*) also correlated with SD-induced growth cessation in *Populus* (Ruonala et al., 2008). *CENL1* is a ortholog of the *TERMINAL FLOWER 1* (*TFL1*), which promotes vegetative growth in *Arabidopsis*, having a negative effect on flowering (Kobayashi et al., 1999). Interestingly, overexpression of PhyA in *Populus* lead to increased *FT* and *CENL1* transcript levels during SD, more particularly after three weeks of exposure (Böhlenius et al., 2006; Ruonala et al., 2008). Thus, in response to photoperiod, *CENL1* may regulate vegetative growth (Ruonala et al., 2008) while the CO/FT module may play a dual role in woody plants, controlling flower induction and vegetative growth cessation (van der Schoot and Rinne, 2011). However, in the latter, the reported time lag between *FT* downregulation in wild type under SD conditions (one week) and the visible downstream effects on growth (three weeks) suggest that, in *Populus*, these genes may not play a direct role in dormancy establishment (Ruonala et al., 2008). Low *FT* expression levels and transport may be a default condition required to

sustain vegetative growth up to dormancy set (van der Schoot and Rinne, 2011).

In addition to photoperiod, global gene expression analysis in woody model plants suggested the involvement of endogenous signalling pathways such as ethylene, abscisic acid (ABA) and gibberellin (GA) signalling in dormancy regulation. Induction of genes related to GA signalling repression (from the *DELLA* gene family) and ethylene biosynthesis, perception and downstream signalling have been observed in *Populus* apical bud samples, up to two weeks after SD treatment (Ruttink et al., 2007). In *Arabidopsis*, activation of ethylene signalling delayed flowering due to a reduction on GA levels and increased GA repressors (Achard et al., 2007). Similarly, the dynamics between both signalling pathways may be involved in early stages of growth cessation and dormancy. The rapid upregulation of GA repressors is consistent with the rapid decrease in cellular GA levels during SD (Olsen et al., 1997), and with the fact that SD-induced growth cessation is delayed in transgenic trees expressing enhanced levels of GAs (Eriksson et al., 2000). In turn, ethylene-insensitive birch (*Betula pendula*) trees showed normal growth cessation under SDs (similar to wild type), but did not develop terminal buds (Ruonala et al., 2006). These results suggested that ethylene is involved in arresting the activity of the shoot apical meristem.

In the same study by Ruttink et al. (2007), the induction of genes related to ABA biosynthesis and signalling followed those of ethylene and GA, at 3-4 weeks after SD exposure. In fact, in birch or *Populus*, ABA levels increase in apical or axilary buds of plants maintained in SD conditions (Welling et al., 1997; Li et al., 2002; Rohde et al., 2002; Welling et al., 2002). However, in *Populus* this increase occurs mainly after growth cessation has started (Rohde et al., 2002; Druart et al., 2007). Hence, while altering ABA levels does not directly appear to influence growth cessation or dormancy development (Welling et al., 1997; Druart et al., 2007), changes in ABA sensitivity seem to affect dormancy (Welling and Palva, 2006;

Karlberg et al., 2010). ABA may have a complex role during seasonal dormancy, which may relate to its role in abiotic stress signalling and response (Gusta et al., 2005). In fact, SD treatments in birch increased freezing tolerance without exposure to low temperatures (Welling et al., 1997; Rinne et al., 1998; Li et al., 2002; Welling et al., 2004). This was also supported in other woody models due to a significant induction of stress-regulated genes observed during dormancy induction, even prior to chilling exposure (Bassett et al., 2006; Druart et al., 2007; Ruttink et al., 2007; Asante et al., 2011). This response may be explained by a decrease in water content and osmotic potential detected during dormancy induction in response to SD (Welling et al., 1997, 2002; Rinne et al., 1998, Welling et al., 2004). Moreover, Druart et al. (2007) also observed that, in response to SD, some changes in poplar cambial metabolome were common to that observed in Arabidopsis cold acclimation. Thus, woody trees depending on SD for dormancy induction may generate a stress-tolerance response prior to cold acclimation.

Although SD is still the best-known prime inducer of activity-dormancy transitions, in some species temperature variations may interact or even replace this signal. As reviewed by Tanino et al. (2010), research in deciduous and coniferous trees reported different but consistent temperature effects during dormancy induction, which was also reflected for the same species growing in different locations. For example, Northern ecotypes, as opposed to Southern, may cease growth, set bud and/or initiate dormancy under specific interactions between low temperature and LDs. In addition, in some species responding to a critical SD photoperiod, warm temperatures may accelerate growth cessation, bud set or increase dormancy depth (Tanino et al., 2010). In some temperate fruit trees insensitive to SD (e.g. some Maloideae species) low temperature showed to be the main inducer of all the morphological and physiological mechanisms leading to dormancy (Heide and Prestrud, 2005). Therefore, it is easy to

assume that the endogenous mechanisms responding to these stimuli may be fine tuned among different species and even ecotypes, to induce seasonal dormancy.

Acquisition of freezing/dehydration tolerance may be initiated during dormancy induction, but subsequent exposure to chilling during late fall and winter may boost cold acclimation to maximum depth, which may vary among different species (van der Schoot and Rinne, 2011). In some woody species like birch, maximum hardiness levels may allow survival (LT_{50}) after exposure to liquid nitrogen temperatures (-196ºC) (Rinne et al., 1998). In peach, the maximum reported value of cold hardiness was -50ºC LT_{50} for bark tissues (Arora et al., 1992). Thus, in addition to growth cessation and dormancy, the timing of subsequent cold acclimation, as well as the depth of cold hardiness are also critical elements in winter survival in temperate and boreal climates. Determining the degree to which temperature mediates all these responses in specific species or genotypes is important to evaluate the impact of future temperature change (Tanino et al., 2010).

In most plants, cold acclimation involves extensive physiological and molecular modifications. Among them, the transcriptional regulation of genes by low non-freezing temperatures leads to the downstream accumulation of cryoprotective proteins and also metabolites involved in cell homeostasis (Thomashow, 1999; Welling and Palva, 2006), like enzymes related to lipid and sugar metabolism, compatible solute biosynthesis, ROS scavenging and metabolic reprogramming (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki 2006; Chinnusamy et al., 2007). Up to now, the best understood regulatory pathway in cold acclimation is mediated by the C-repeat binding factor (CBF)/dehydration responsive element binding factor 1 (DREB1) family of transcription factors (TFs). These TFs are rapidly induced by cold, leading to the activation of target genes with a determinant role in the acquisition of freezing tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006; Medina et al., 2011). In addition, CBF TFs were recently

suggested to regulate other developmental processes such as vegetative growth and senescence, in *Arabidopsis* (Achard et al., 2008; Sharabi-Schwager et al., 2010). The evidences that *CBFs* transcription may also be regulated by the circadian clock and light, even under normal growth conditions (Fowler et al., 2005; Franklin and Whitelam, 2007; Bieniawska et al., 2008) support the central role of these genes in the environmental control of plant development. CBF TFs have been studied in a wide variety of herbaceous and woody plants (Welling and Palva, 2006; Medina et al., 2011), demonstrating the conservation of this cold signalling pathway among distantly related species. In some perennial plants, *CBF* genes showed to be differentially regulated by photoperiod (El Kayal et al., 2006; Welling and Palva, 2008). In addition, overexpression of a peach *CBF* gene in apple (SD-insensitive) induced dormancy under SD conditions (Wisniewski et al. 2011). Together, these results suggest a critical role of the CBF regulon in dormancy regulation in the perennial context.

5.2. Dormancy release

Understanding the mechanisms controlling dormancy release is of great importance in horticultural crops, since it can affect the extent of the growing season and also plant productivity. As previously mentioned, chilling exposure during winter has a critical effect in this process due to plant specific CRs. Chilling-induced release from endodormancy is assumed as a local phenomenon because in dormant tissues the effect of chilling is not imported from other parts of the plant. Restoring plasmodesmata functionality in the buds may be one of the targets for the mechanisms controlled by chilling (van der Schoot and Rinne, 2011). Catabolism of sucrose and fats is also thought to occur during this stage, providing energy for dividing cells during the early stages of reactivation, while significant photosynthetic activity is still not observed (Druart et al., 2007).

Currently the molecular components mediating CRs in woody plants have been receiving increased attention. In poplar, long-term chilling accumulation in vegetative buds potentially promotes the production of bioactive GAs, by inducing the expression of genes related to GA biosynthesis (Druart et al., 2007; Karlberg et al., 2010; Rinne et al., 2011). Recently, Rinne et al. (2011) observed that bioactive GAs induce the expression of specific genes from a glucan hydrolase family, which may be recruited to remove the callose deposited in the plasmodesmata of dormant bud cells. Thus, during the transition to growth-promotive temperatures, functional pipelines are available for the transport of the vegetative growth promoters (such as FT or CENL1), within the apex (Rinne et al., 2011). Additionally, GAs may also function during dormancy release by increasing the degradation of growth-repressive DELLA proteins, which accumulate during cold acclimation or SD-induced growth cessation (van der Schoot and Rinne, 2011)

The existence of genes with dual roles in the regulation of flowering and dormancy has been reported, for example, for the *CO*/*FT* regulatory module in poplar (as previously mentioned in 5.1). In this way, a comparison between the chilling control of dormancy break in woody plants and the vernalization-induced floral promotive pathways in annual plants is a tempting approach to identify new regulators of the former pathway (Horvath, 2009). In *Arabidopsis*, genes like *FRIGIDA* (*FRI*), *VERNALIZATION INSENSITIVE 3* (*VIN3*) and FLOWERING LOCUS C (FLC) are involved in the vernalization pathway. FRI is involved in vernalization requirements, repressing flowering to a degree that makes ineffective the influence of other favourable conditions. This protein promotes the accumulation of *FLC*, which encodes a MADS-box protein that, due to its negative regulation of floral pathway integrator genes like *FT* (Simpson and Dean, 2002) represses flowering in a quantitative way. Vernalization results in the reduction of FLC, in part due to the action of

VIN3, which is induced after prolonged exposure to cold temperatures. In *Populus*, the expression of an *FLC*-like gene was shown to decline in dormant buds during cold exposure, being associated with dormancy release (Chen and Coleman, 2006). Still, more detailed evidences are needed to prove the involvement of this specific pathway in dormancy maintenance and release in woody plants.

Interestingly, an important contribution regarding the genetic control of CRs came from comparative studies between deciduous and evergrowing (*evg*) peach genotypes. The *evg* mutant is a naturally occurring genotype identified in Southern Mexico, which shows dormancy-impaired development, maintaining continuous terminal growth and persistent leaf production when exposed to dormancy-inductive conditions (Rodriguez et al., 1994). This trait also associates with a reduced level of cold hardiness, when compared to deciduous genotypes (Arora et al., 1992). The *EVERGROWING* (*EVG*) locus is located on the terminal portion of LG1 in peach, overlapping a major QTL for CRs (Fan et al., 2010), and is associated with both mutant and deciduous genotypes (Bielenberg et al., 2008). However, six tandemly-duplicated genes within this locus were found to be expressed in deciduous, but not in the non-dormant *evg* genotype, due to a major genomic deletion (Bielenberg et al., 2008). These genes belonged to the MIKC-type MADS-box gene group and were named *DORMANCY ASSOCIATED MADS-box* genes (*DAM*). DAMs are phylogenetically related to the *Arabidopsis* AGAMOUS-LIKE 24 (AGL24)/ SHORT VEGETATIVE PHASE (SVP) type of MADS-box transcription factors, which are primarily expressed in vegetative tissues, regulating growth or the transition from vegetative to reproductive meristems (Liu et al., 2009). Interestingly, in *Arabidopsis*, SVP and FLC may associate in a floral-repressor complex (Li et al., 2008).

Peach *DAM* genes are differentially regulated during activity-dormancy cycles in peach (Li et al., 2009). *DAM1*, *DAM2* and *DAM4*

expression pattern is more closely associated with growth cessation and bud set while *DAM3*, *DAM5* and *DAM6* are winter expressed (Li et al., 2009), being repressed after continuous chilling exposure in floral and vegetative buds (Jiménez et al., 2010). More specifically, expression of *DAM5* and *DAM6* showed a straight negative correlation to the acquisition of cultivar specific chilling requirements (Jiménez et al., 2010, Yamane et al., 2011). These genes also showed to be downregulated in vegetative buds after treatment with a dormancy-breaking reagent (Yamane et al., 2011). Therefore, while the precise function of each one of the peach *DAM* genes still needs to be determined, *DAM5* and *-6* may act as growth-inhibitor signals during seasonal dormancy (Yamane et al., 2011).

DAM-like genes have been associated with endodormancy regulation, not only in other species from Rosaceae (Mazzitelli et al., 2007; Yamane et al., 2008; Sasaki et al., 2011) but also in poplar (Ruttink et al., 2007) and leafy spurge (*Euphorbia esula*) (Horvath et al., 2008), demonstrating the existence of a conserved mechanism related to *DAM* function in perennials. Heterologous overexpression of leafy spurge *DAM1* (putatively involved in dormancy induction) in *Arabidopsis* decreased endogenous *FT* transcript levels and delayed growth (Horvath et al., 2010). Moreover, overexpression of Japanese apricot DAM6 in poplar induced growth cessation and bud set under non-inductive conditions, also affecting the expression of an *FT* ortholog (Sasaki et al., 2011). These results support the previous hypothesis of an inhibitory effect of DAM genes on growth. Interestingly, the promoter regions of several of these *DAM* genes contain putative *cis*-regulatory elements related to the CBF-mediated cold signalling pathway.

Transcriptomic analysis in horticultural crops such as peach, apricot, grapevine (*Vitis riparia*) and raspberry revealed new candidate genes with differential expression between endo and ecodormant bud stages (Leida et al., 2010; Yamane et al., 2008; Mathiason et al., 2009; Mazzitelli et al.,

2007). These included transcripts related to gibberellin metabolism, stress resistance, cell wall modification, cellular transport and signal transduction, among others. *DAM5* and/or *DAM6* genes were also represented in the cDNA libraries for endodormant buds obtained for peach and apricot (Yamane et al., 2008; Leida et al., 2010, 2011). Further detailed validation of some of these candidates may provide new interesting markers for dormancy-activity transition in these species. Recently, Leida et al. (2011) analyzed the expression pattern of several candidate genes for bud dormancy-activity transitions in ten different cultivars with varying CRs. At a specific developmental stage, when some varieties had already fulfilled their chilling needs and acquired growth competence, some of these genes showed a striking decrease in transcript accumulation in comparison to high CR cultivars (Leida et al., 2011). *DAM4*, *-5* and *-6* were among these genes, once again confirming their relevance as candidates for dormancy regulation.

Although growth ability is restored after endodormancy break, growth resumption will only occur in the presence of growth promotive conditions, mainly related to warm conditions in spring, but also, in some cases, warm spells still during winter. As previously mentioned, HR may regulate this process in some extent. However, up to now studies regarding the molecular mechanisms underlying this control are scarce. Nevertheless, several studies associate endodormancy break with a decrease in hardiness levels (Kalberger et al., 2006), which in a way could associate to temperature increase. In chestnut (*Castanea sativa*), the phased transcription of circadian oscillator genes is disrupted during winter dormancy (Ramos et al., 2005), which was confirmed to be mainly due to low temperature response (Ibañez et al., 2008). Therefore, ecodormancy break may be also related to the reestablishment of the circadian clock.

5.3. Epigenetic regulation of dormancy induction and release

Epigenetics is an emerging area of interest in life science research. Due to their role in chromatin remodelling and subsequent control of gene expression, epigenetic marks such as histone modifications and DNA methylation, as well as their main regulator elements, have been subject of extensive studies. Epigenetic mechanisms have a key role on plant plasticity in response to environmental stimuli (He et al., 2010, Santos et al., 2011). Thus, the major transcriptomic and metabolic reprogramming reported in buds or even meristems during dormancy induction, maintenance and release (e.g. Yakovlev et al., 2006; Ruttink et al., 2007; Rohde et al., 2007; Druart et al., 2007; Asante et al., 2011; Leida et al., 2010; Mathiason et al., 2009; Yamane et al., 2008) strongly suggests the involvement of epigenetic regulation.

The differential expression of genes directly or indirectly involved in histone modifications has been recently reported (Druart et al., 2007; Karlberg et al., 2010; Santamaría et al., 2011). In *Populus*, induction of histone-modifier genes often related to transcriptional repression or members of repression-protein complexes with similar functions occurred during dormancy establishment (Druart et al., 2007; Karlberg et al., 2010). The downregulation of transcripts encoding a DNA glycosylase also showed to occur during dormancy induction, which suggests the occurrence of an increased DNA methylation (Karlberg et al., 2010). In agreement, in chestnut vegetative buds or in strawberry (*Fragaria* x *ananassa*) leaves, an increase in global DNA methylation was observed during dormancy induction (Santamaría et al., 2009; Zhang et al., 2011). These modifications may be in agreement with the lower cellular activity during dormancy stages, since they are usually related to inactive chromatin. Additionally, alterations in histone acetylation patterns favouring transcription initiation were observed during endodormancy release in potato tubers (Law and Suttle, 2004). Nevertheless, the simple fact that, during dormancy, plants

are also able to induce cold acclimation suggests that the previous observations may be masking more complex interactions between epigenetics and transcription. Moreover, induction or repression of a given gene by epigenetic factors is dependent on site-specific modifications occurring in the corresponding regulatory regions (e.g. promoters and introns [Baek et al., 2011; Shibuya et al., 2009; Liu et al., 2004]), and the analysis of specific targets is thus crucial.

Recent studies suggested the role of histone modifications on the regulation of *DAM* genes in leafy spurge and peach (Horvath et al., 2010; Leida et al., 2011). In the latter, the downregulation of *DAM6* in two cultivars with distinct CRs during dormancy release, correlated to the decrease of specific histone modifications related to active chromatin within the translation start site region. In turn, an increase in other histone modifications related to inactive chromatin was detected in a broader level (promoter, translation start site and intronic regions) (Leida et al., 2011). These results demonstrate the role of chromatin modifications in the regulation of a candidate gene controlling endodormancy. Considering that vernalization in *Arabidopsis* also leads to *FLC* downregulation due to similar epigenetic modifications (Sung and Amasino, 2004), the similarities between dormancy in perennials and flowering plants are again highlighted (Leida et al., 2011).

6. Challenges and opportunities to understand blooming time in Rosaceae fruit trees

A major challenge for genomic studies in fruit trees is to identify the functions of candidate genes and use this knowledge to improve economically important agronomic and quality traits. Considering the increasing knowledge generated from high resolution mapping of QTLs for blooming time and CRs, the candidate gene approach is currently a

valuable opportunity to bridge the gap between genomics and functional analysis of genes possibly related to this traits. *DAM* genes are a good example of this connection. The previously mentioned global transcriptomic studies already provided an extensive list of candidate genes related to dormancy break. In addition, the recently sequenced genomes from peach (www.rosaceae.org, 2010), apple (Velasco et al., 2010) and even strawberry (Shulaev et al., 2011) provide a major advantage for future studies. Besides determining the patterns of seasonal expression, candidate genes should be validated in terms of putative function as well as determining the upstream regulators related to dormancy maintenance. Comparative studies, either at the genomic, epigenomic or functional level, between cultivars with contrasting levels of CRs may also provide a better understanding of this subject.

A demarked difference between some temperate perennial trees, such as most fruit tree crops and conifers and other model trees (*Populus* and birch) is the noticeable tendency for a delay between flower initiation and emergence (Battey, 2000). Although the mechanisms for dormancy regulation in vegetative and flower buds may partially overlap, the complex structure of the flower bud, with multiple differentiated organs, may imply the existence of other levels of regulation. For example, in opposition to the aforementioned definitions of dormancy in perennials, the endodormancy in peach flower buds was defined as ‘a combination of ongoing cell division, enlargement, and differentiation that results in organogenesis during the entire dormancy period’ (Reinoso et al., 2002).

Genes involved in flower induction (*FT*, *CO*, *FLORICAULA/LEAFY*) or repression (*TFL1*), have been isolated from Rosaceae trees (Silva et al., 2007; Hanke et al., 2007; Hattasch et al., 2008; Kotoda et al., 2010). The function of some of these genes (like *FT* or *TFL1*) in these species has been demonstrated by genetic transformation, in homologous or heterologous systems (Hanke et al., 2007; Hattasch et al., 2008; Kotoda et al., 2010;

Tränkner et al., 2010). Interestingly, *FT*, *CO* and *TFL1*, in addition to other floral-related genes from apple, showed an interesting pattern of expression in vegetative buds during blooming of adjacent buds in early spring and flower induction in late spring (Hattasch et al., 2008). Still, the involvement of these genes during endo and ecodormancy induction and release, and possible correlation to CRs or HRs still remains poorly studied in these species.

Certain aspects of floral organ development, such as stamen maturation, have been associated with endodormancy release (Julian et al., 2011). Considering that the floral organs resume their development more rapidly after endodormancy break (Reinoso et al., 2002), it would be interesting to study the role of floral homeotic genes (involved in organ identity) during this re-activation and possible upstream regulators associated with CRs. Several *MADS-box* genes associated to the ABCDE model of floral organ identity have been already identified in fruit trees (Hanke et al., 2007). In addition, given the conservation occurring between flower induction in annual models and seasonal dormancy in perennials, it would be interesting to analyse if the mechanisms activated during anthesis in annuals also occur in fruit tree species. For example, GAs produced in anthers were shown to be involved in promoting the expansion of the corolla in *Arabidopsis* (Weiss and Halevy, 1989; Hu et al., 2008). It would be interesting to study if and how this mechanism could fit in the context of ecodormancy break in fruit trees.

7. Main goals and thesis outline

The current knowledge regarding dormancy transitions in woody plants evidences the role of several interacting signalling pathways, which are fine tuned in order to synchronize growth with favourable environmental conditions. A proposed model, depicting some of the most consistent genes and environmental factors involved in dormancy regulation in perennials, is represented in Figure 3. Although low temperature is a major signal in this process, the involvement of well-known cold signalling factors for cold acclimation, like the C-repeat Binding Factor (CBF) family of transcription factors is still poorly studied in woody trees, particularly for its role along seasonal dormancy.

The main goal of the current thesis was to identify and elucidate the functional role of new *CBF*-like genes in a relevant fruit tree. Almond was chosen as a model for other Prunoideae considering its lower chilling requirements (Egea et al., 2003; Alonso et al., 2005, 2010) and its wide adaptability to different climatic conditions (Alonso et al., 2005), probably related to the high genetic variability.

This work was initiated in 2007, three years before the peach genome became publicly available. Therefore, we defined a cloning strategy to identify and sequence CBF-like genes in almond (*PrdCBF*s), as well as the corresponding regulatory promoter regions. This work is described in Chapter II. Additionally, this Chapter describes the *PrdCBFs* gene localization in the *Prunus* reference map and the analysis of their gene expression in response to low temperatures.

In Chapter III we report the functional characterization of these genes, by testing their role as transcription factors. This was performed by transient expression in protoplasts and overexpression in *Arabidopsis* plants.

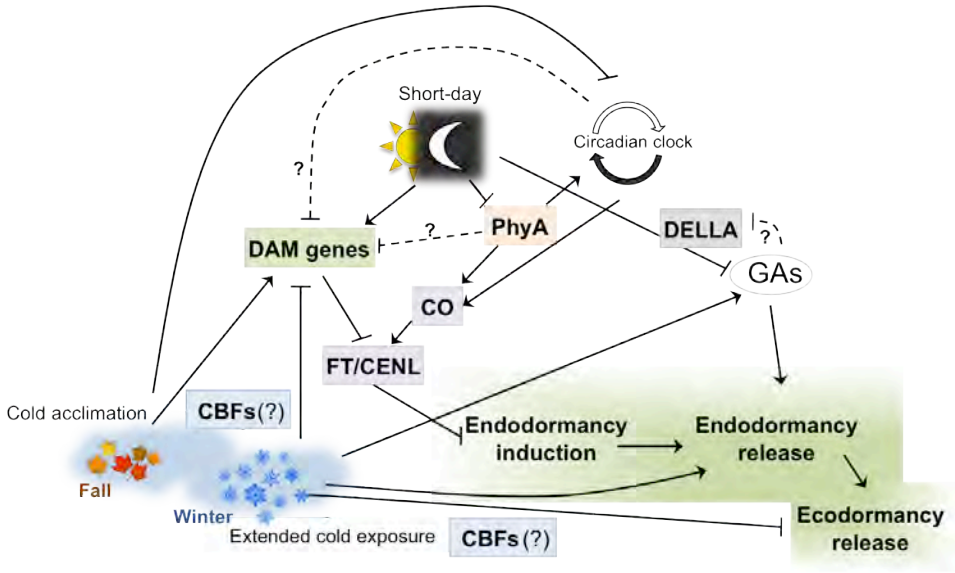

Figure 3. Schematic model for the regulation of bud dormancy by low temperature and day length in perennial plant species (adapted from Horvath, 2009). Short-day photoperiod (SD) and low temperature perception leads to the activation of DORMANCY ASSOCIATED *MADS-box* genes (DAM) (Li et al., 2009; Horvath et al., 2010; Yamane et al., 2011). Additionally, the negative regulation of phytochrome A (PhyA)-mediated signalling during SD (Kozarewa et al. 2010), and further cold acclimation may affect the circadian clock (Ibañez et al., 2008). DAM products induced during this stage most likely repress *FLOWERING LOCUS T* (*FT*) and/or *CENTRORADIALIS-LIKE1* (*CENL*) growth promoter genes. Repression of FT may also be imposed by the reduction of CONSTANS (CO) during SD perception. Moreover SD perception may also decrease the levels of gibberellins (GAs) (Olsen et al. 1997) due to the induction of growth repressive DELLA-proteins (Ruttink et al., 2007). Thus, repression of FT/CENL and accumulation of DELLA proteins may lead to endodormancy in perennial buds. Extended cold temperatures (chilling accumulation) may repress the expression of DAM genes (Yamane et al., 2011) and induce the expression of GA biosynthetic genes (van der Schoot and Rinne, 2011), restoring growth ability (according to specific chilling requirements) and/or activating growth during ecodormancy break (since low temperatures may still repress growth and development after endodormancy). CBF transcription factors are candidate players in seasonal cold acclimation and also activators of DAM gene expression during dormancy induction.

To assess the role of *PrdCBF*s during winter dormancy, we describe in Chapter IV a gene expression analysis of *PrdCBF*s in flower buds and shoots from adult almond trees, during fall and winter stages. This study was conducted along with the expression analysis of additional candidate

marker genes for flower development, during two consecutive years. These latter genes were helpful to follow the mechanisms related to flower bud maturation.

Considering the seasonal expression pattern obtained for one specific *PrdCBF*, and in order to elucidate the putative role of DNA methylation during dormancy transitions in flower buds, we have additionally determined the DNA methylation pattern for a specific promoter region of this gene. This work is further reported in Chapter V.

Finally, in Chapter VI we discuss the importance of the results obtained and how further studies will allow investigating the different hypothesis rose throughout this work.

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CHAPTER II

Cloning and Characterization of Two Almond *CBF* Genes Involved in Cold Response

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Summary

Low temperature plays a crucial role in seasonal development of woody plants, regulating the extent of dormancy and initiation of vegetative and reproductive periods. Still, little is known regarding the mechanisms underlying cold signalling in these plants, particularly fruit trees. Given its high genetic variability and adaptability to different climatic conditions, almond (*Prunus dulcis* Mill.) is an interesting model to understand the mechanisms regulating low temperature sensing in fruit trees. In this paper we report the cloning and characterization of two genes belonging to the C-repeat binding factor (CBF) family of transcription factors (*PrdCBF1* and *PrdCBF2*). Southern blotting analysis showed that this family is composed of at least four members. In almond shoots propagated *in vitro*, transcript accumulation of *PrdCBF1* and *PrdCBF2* was rapidly induced by low temperature, suggesting an involvement in cold acclimation. In addition, induction of both *PrdCBF*s by cold was higher towards the end of the day, which agreed with the expression pattern of *PrdDehydrin1*, a predicted CBF target gene. Furthermore, *PrdCBF*1 and *-2* were also transiently induced by ABA and drought treatments. Considering the bin mapping analysis that correlated *PrdCBF*s and *PrdDHN1* (respectively in linkage groups 5 and 7) with two different QTLs controlling blooming time, it is relevant to perform further association studies that may validate their effect on this trait.

1. Introduction

Cold stress is a major environmental stress, limiting crop productivity in cultivated areas. Plant species may have different levels of susceptibility either to chilling (0-15ºC) or freezing (<0ºC). Nevertheless, they can increase their freezing tolerance upon exposure to low non-freezing temperatures, in a process known as cold acclimation or cold hardening (Levitt, 1980).

Cold acclimation involves extensive physiological and molecular modifications, such as transcriptional modulation of specific genes and accumulation of cryoprotective proteins and other metabolites involved in cell homeostasis (Thomashow, 1999; Nakashima and Yamaguchi-Shinozaki, 2006). The C-repeat binding factor (CBF)/dehydration responsive element binding factor 1 (DREB1) family of transcription factors (TFs) mediates one of the best-studied mechanisms of low temperature signalling in plants (Yamaguchi-Shinozaki and Shinozaki, 2006; Medina et al., 2011). These TFs were first identified in *Arabidopsis* after a yeast-one hybrid screening towards the conserved CRT/DRE response element, present in the promoters of many cold- and drought-induced genes involved in stress tolerance (Stockinger et al., 1997; Liu et al., 1998). CBFs contain a highly conserved AP2/ERF (APETALA 2/Ethylene Responsive Factor) domain, which acts as a DNA-binding motif and an acidic C-terminal sequence conferring transactivation activity (Stockinger et al., 1997; Medina et al., 2011). Transcriptional induction of *CBF*s occurs rapidly after temperature decrease and this is closely followed by the increased expression of their target genes (the CBF regulon), which play a fundamental role in cold acclimation. Several reports have shown that the CBF signalling pathways may also integrate additional cues related to changes in the surrounding environment, such as ABA, drought or mechanical stress (Gilmour et al., 1998; Knight et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006).

Additionally, light quality and the circadian clock were shown to modulate *CBF* expression (Fowler et al., 2005; Franklin and Whitelam, 2007; Bieniawska et al., 2008).

The CBF-mediated cold signalling pathway seems to be a conserved mechanism among distantly related species. CBF TFs have been targeted in a wide variety of herbaceous and woody plants (Welling and Palva, 2006; Medina et al., 2011), deepening the knowledge related to variations in freezing tolerance occurring within different plant species throughout evolution. Factors like the expression level of different *CBF* members and the size and diversity of the CBF regulon have been suggested as crucial in this natural variability (Zhang et al., 2004; Hannah et al., 2006; Champ et al., 2007; Badawi et al., 2007; Carvallo et al., 2011). Concerning woody species, the involvement of *CBF* genes in cold acclimation has been studied in sweet cherry (*Prunus avium*) (Kitashiba et al., 2004), birch (*Betula pendula*) (Welling and Palva, 2008), poplar (*Populus trichocarpa*) (Benedict et al., 2006), eucalyptus (*E. gunni and E. globulus*) (El Kayal et al., 2006; Gamboa et al., 2007; Navarro et al., 2009), grape (*Vitis* spp.) (Xiao et al., 2006; 2008), citrus (*Poncirus trifoliata* and *Citrus paradisi*) (Champ et al., 2007), highbush blueberry (*Vaccinium corymbosum*) (Polashock et al., 2010), dwarf apple (*Malus baccata*) (Yang et al., 2011), apple (*Malus* x *domestica*) and peach (*Prunus persica*) (Wisniewski et al., 2011).

Perennial trees from temperate or boreal environments are frequently exposed to chilling and freezing conditions, particularly during winter dormancy. In fact, maximal cold hardiness levels, which in some deciduous trees can reach liquid nitrogen temperatures (Rinne et al., 1998), are achieved during seasonal acclimation, by a sequential process induced by photoperiod and/or temperature conditions (Weiser, 1970; Welling and Palva, 2006; Tanino et al., 2010). In several woody plants, variations in *CBF* expression have been related to photoperiod (El Kayal et al., 2006) and dormancy (Welling and Palva, 2008), probably indicating that *CBF*

regulation in these species may entail different levels of complexity, as compared to herbaceous plants.

Dehydrins (DHNs) are a sub-group of the late-embryogenesis abundant (LEA) proteins, widely studied in woody plants due to their predicted role in protection against cellular dehydration and cold acclimation (Welling and Palva, 2006). The precise function of these proteins remains to be determined, although several studies elucidated their cryoprotective and antifreeze properties (Wisniewski et al., 1999, Rorat, 2006). Seasonal patterns of *DHN* expression appear to be a common feature in woody plants (Welling and Palva, 2006), namely in fruit species (Yamane et al., 2006; Bassett et al., 2009; Garcia-Bañuelos et al., 2009). In peach, *PpDHN1* was isolated from cold acclimated bark tissues and its expression was mainly induced by cold (Wisniewski et al., 2006). The presence of DRE/CRT motifs in the promoter region of several cold-regulated *DHN*s suggests that these are predicted players in the CBF-mediated signalling pathway (Puhakainen et al., 2004; Wisniewski et al., 2006; Bassett et al., 2009).

In addition to the role in seasonal cold acclimation, the prolonged exposure to cold temperatures during winter also determines the timing of dormancy break (Metzger, 1996). In Rosaceae fruit trees this timing affects the extent of the growing season and also crop production. Thus, understanding the mechanisms underlying cold signalling and response in these plants is crucial. Almond (*Prunus dulcis* Mill.) is the earliest fruit tree to bloom in winter/spring, having low chilling requirements for dormancy break (Arús et al., 2009). However, this species has a wide adaptability to different climatic conditions (Alonso et al., 2005) associated with its high genetic variability, thus being an interesting model to study low temperature response in Prunoideae. Considering that the CBF-mediated signalling pathway is still poorly understood in this species and in general within the *Prunus* genus, in this Chapter we describe the identification and functional characterization of two almond *CBF* genes.

2. Materials and Methods

2.1 Plant material

Almond shoots were obtained from *in vitro* axillary budding of adult cv. Verdeal. Shoots were subcultured every three weeks on micropropagation medium as described by Miguel et al. (1996) and maintained under 16h/8h photoperiod at 22/20ºC. Genomic DNA was extracted as described by Martins et al. (2003). RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA), treated with RNAse-free DNase I (Qiagen) and quantified using NanoDrop (Thermo Scientific, Wilmington, DE, USA).

2.2 Identification of PrdCBF-*like sequences*

To clone CBF-like sequences from almond, specific primers CBF-F and CBF-R (Table 1) were designed based on the nucleotide sequence of sweet cherry *PaCBF1* (accession n. AB121674). Two week-old propagated shoots were transferred to 5ºC chamber during 12h and collected at selected intervals and frozen in liquid nitrogen. Four micrograms of total RNA pool obtained from the cold treatment was used for cDNA synthesis with SuperScript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) following manufacturers' instructions. Polymerase chain reaction (PCR) mixture was prepared in 15µL volume using 1µL cDNA as template and 1U GoTaq® DNA Polymerase (Promega Corp., Madison, WI, USA) according to manufacturers' instructions. Samples were analyzed by electrophoresis on 1% agarose gel. The predicted amplicons were excised from gel and purified using the Wizard® SV Gel Clean-Up System (Promega), cloned into pCR2.1 (Invitrogen) following manufacturers' instructions and sequenced (STABVida, Portugal).

Table 1: Sequences of the primers used for molecular cloning of *PrdCBF1*, *PrdCBF2* and *PrdDHN1*, RACE-PCR, inverse PCR and mapping analysis.

Genes	Experiments	Primer	Primer sequence
PaCBF	Gene cloning	CBF-F	5'-GCCCCAGTCGAGTTTGTTGTC-3'
		CBF-R	5'-AGCATTGCGATGGAGAAAGAAG-3'
PrdCBF1	3' RACE and Mapping	CBF-3F	5'-CAAGTGGGTGTGTGAAATGAG-3'
	5' RACE	CBF1-5R1	5'-AATGAAAATCAAATTACAACGGTC-3'
		CBF1-5R2	5'-TGGAGAAACTCCACAATTTGACATC-3'
	iPCR	iPCR1-F	5'-GCTTGTCTGCCTCAACTTCC-3'
		iPCR1-5R1	5'-GGACCAAGAAGCCCTTAGAGTC-3'
		iPCR1-5R2	5'-CGGACAACGAACTTGACTCG-3'
	Mapping	CBF1-5R3	5'-CCAACAGGGACACAAAGGAAGCCAAC-3'
PrdCBF2	5' RACE	CBF2-5R1	5'-ACCCATGTCATCCAAATTCATGTC-3'
	5'RACE and Mapping	CBF2-5R2	5'-CAAACAATAATCGCTCGCACA-3'
	iPCR and Mapping	CBF2-5F	5'-ACTTTGCTGACTCCGCGTTG-3'
	iPCR	iPCR2-5R2	5'-GGGTCGGAAAGTTGAGACAAG-3'
		iPCR2-5R1	5'-AAGAAGGTCCCTGAGTCGTG-3'
-	RACE	Adp-R	5'-CCACGCGTCGACTAGTAC-3'
		AdpPoliT-3R	5'-CCACGCGTCGACTAGTACT$_{(18)}$VN-3'
		AdpGGI-5F	5'-GCCACGCGTCGACTAGTACCGGGGIIGGII GGGIIG-3'
PrdDHN1	Mapping	PrDHN1-F	5'-CGTTGTGGTATCTTACGGCAC-3'
		SalDHN1-R	5'-CCGTCGACGCAACGAATCTACACCCAAA-3'

2.3 Rapid Amplification of cDNA Ends

Full-length coding sequences were obtained by Rapid Amplification of cDNA Ends (RACE-PCR) according to Frohman et al. (1988), with minor modifications. Messenger RNA was purified using the PolyATract® mRNA Isolation System III (Promega) from the cold-induced total RNA pool. Sixty nanograms of this mRNA was then used for cDNA synthesis using the SuperScript® First-Strand Synthesis System (Invitrogen). The 3'-RACE was

performed according to Borson et al. (1992), using a specific forward primer (CBF-3F) for both genes. For 5'RACE, single stranded cDNA was synthesized using CBF1-5R1/CBF2-5R1 gene specific primers and modified with the addition of an oligo(C) tail (anchor region), as described by Bekman et al. (2000). For the amplification of 5' cDNA ends, PCR was performed using gene specific reverse primers CBF1-5R2/CBF2-5R2 and a forward primer (AdpGGI-5F) specific for the anchor region. All amplification reactions were performed using 0.4U Phusion® High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland) according to manufacturers' instructions. Amplification products were separated by electrophoresis on 1% agarose gels and fragments of interest were purified, cloned and sequenced as mentioned in 2.2. For each gene, several 5'RACE products were sequenced and analyzed and the first nucleotide of the longer 5' clone was considered as the beginning of the mRNA. All the primers used for 3'- and 5'-RACE are listed on Table 1.

2.4 Promoter cloning

The promoter regions of the identified *PrdCBF*s were targeted using inverse PCR (iPCR), as described by Ochman et al. (1988). Specific primers were designed for each coding sequence 5' and 3' ends, facing opposite orientations in a way that extension could occur outwards the known genomic regions (Figure 1). *In silico* restriction analysis was performed to search for enzymes that could cut at least once, downstream the primer located towards the 3' end. Two micrograms of almond genomic DNA was digested with 10U *Nco*I and *Not*I for 6h in 60µL total volume at 37ºC, followed by enzyme inactivation by 10min incubation at 65ºC. DNA circularization was carried out during 16h at 14ºC using 15µL of the digestion reaction in 100µL ligation reaction with 4U T4 DNA ligase (Invitrogen). Eight microliters of each ligation reaction was used in a first PCR round in 100µL volume with either iPCR1-F/iPCR1-5R1 or PdCBF2-

5F/iPCR2-5R1 primer combinations (Table 1). PCR was performed as follows: 5min incubation at 95ºC, followed by 30 cycles of 90sec at 95ºC, 60sec at 56ºC and 3min at 72ºC and a final extension step of 5min at 72ºC. After this, a nested PCR was prepared with 4µL of the previous reaction mixture using the same forward primers and the nested reverse primers iPCR1-5R2 or iPCR2-5R2 (Table 1). Amplification was performed as above, decreasing the cycling extension time to 150sec. Amplification products were analyzed by electrophoresis on 1% agarose gels and fragments of interest were cloned and sequenced as mentioned in 2.2. The 5' genomic end was located immediately downstream the enzyme recognition site.

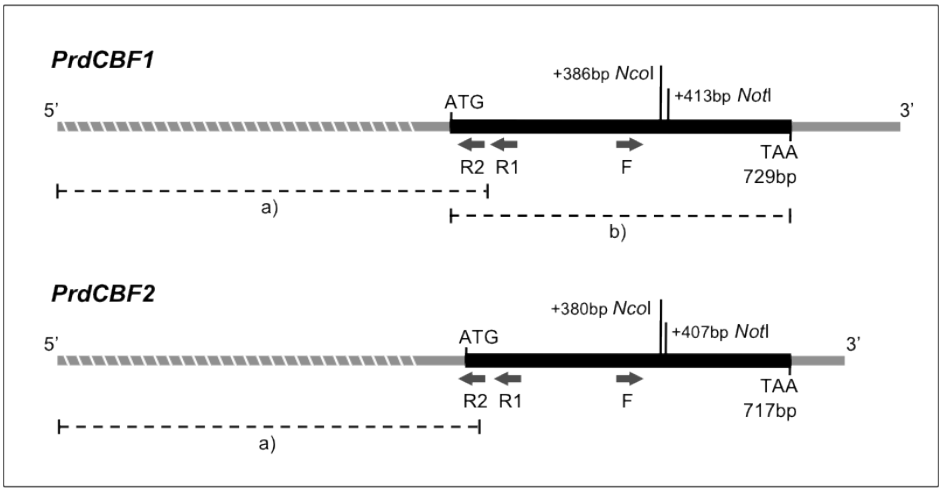


Figure 1. Schematic representation of *PrdCBF1* and *PrdCBF2* coding regions (black line), 5' and 3' untranslated regions extended with RACE-PCR (gray line) and the promoter region identified by iPCR (striped gray line). The locations of the restriction enzymes used for iPCR are indicated. Gray arrows represent the primers used for iPCR: F - forward primer, R1 - reverse primer, R2- reverse primer for nested PCR. The regions used as probes for Southern-blot hybridizations are represented as dashed lines for (a) promoter region used for high stringency analysis, (b) *PrdCBF1* coding region used for medium stringency analysis. bp – base pairs.

2.5 In silico *sequence analysis*

All sequences were edited using EditSeq and *in silico* restriction analysis was performed using MapDraw, both from Lasergene software package (DNASTAR Inc., Maddison, WI, USA). Pairwise and multiple sequence alignments were made using ClustalW (Larkin et al., 2007) and phylogenetic analysis was performed by the neighbor-joining method using MEGA4 software (Tamura et al., 2007). *PrdCBF*s promoters were analyzed using databases of *cis*-regulatory elements available online (PLACE, Higo et al. [1999]; PlantPAN, Chang et al. [2008])

2.6. Mapping analysis

Mapping analysis was performed using the bin mapping strategy described by Howad et al. (2005) on the *Prunus* reference map, constructed from an almond (cv. Texas) x peach (cv. Earlygold) F2 population. Genomic DNA from the parental cultivars, the F1 hybrid and the six F2 selected individuals (5, 12, 23, 30, 34 and 83) were kindly provided by Dr. Pere Arús (IRTA, Barcelona). *In silico* sequence analysis was performed to search for restriction enzymes that could generate cleaved amplified polymorphic sequence markers (CAPS). For both *PrdCBF*s this was performed using the 3' end of each cDNA in order to include the 3'UTR region and the following enzymes were selected: *Hae*III, *Bae*I, *Not*I, *Sac*II and *Taq*I. For *PrdDHN1*, the promoter region was analyzed and *Xba*I was selected since it contained one recognition site for peach sequence and none for almond. Fragments were amplified by PCR for all nine genotypes, using the correspondent primer pairs (CBF-3F and CBF1-5R3 for *PrdCBF1*; CBF2-5F and CBF2-5R2 for *PrdCBF2*; PromDHN1-F and SalDHN1-R for *PrdDHN1,* Table 1). PCR was performed with GoTaq polymerase (Promega) according to manufacturers' instructions in 30 amplification cycles. Five microliters of each PCR reaction were used for restriction analysis. Digestions were performed using 2U of each enzyme during 6h-12h at the corresponding

incubation temperature. Restriction reactions were analyzed by electrophoresis on 1.2% agarose gels. Mapping was performed by matching the joint genotype of each marker, generated for the F2 individuals, to that of the set of bins obtained in the framework map (Supplementary Table 1) (Howad et al., 2005).

2.7 Southern blotting

Almond genomic DNA was obtained from *in vitro* almond shoots as mentioned above and quantified by gel electrophoresis with λ DNA (Fermentas Inc., Ontario, Canada). Ten micrograms of DNA were digested with *Eco*RI (Fermentas), *Eco*RV and *Sst*I (Jena Bioscience, Jena, Germany) and separated on a 0.8% agarose gel at 1.4V/cm during 12h-16h. Digested DNA was blotted onto Hybond N^+ membrane (GE Healthcare, Uppsala, Sweden) by alkaline transfer (Sambrook et al., 1989). Probes (Figure 1) were labelled with [α-^{32}P] dATP by random priming using Klenow Fragment (New England Biolabs, Ipswich, MA) according to manufacturers' instructions. Membranes were pre-hybridized for 2h in Church buffer (Church and Gilbert, 1984) at 62 or 66ºC (for medium or high stringency, respectively) and hybridized overnight with Church buffer supplemented with approximately $2x10^7$ cpm mL^{-1} of denatured probe. After hybridization, the membranes were washed twice in 2xSSC for 5 min and once in 2xSSC + 0.1%SDS for 10min, at 62/66ºC. A final wash was performed in 1xSSC + 0.1%SDS for 10 min, at room temperature. Membrane exposure to Amersham Hyperfilm™ MP (GE Healthcare) was carried out at -80ºC during 12h and 48h. Membranes were used for multiple hybridizations by stripping the probe in 0.1% SDS at 68 °C during 2h.

2.8 Stress treatments

For gene expression analysis of both *PrdCBF*s during cold shock treatment, two week-old *in vitro* almond shoots were adapted to 12h/12h

photoperiod during five days at 22ºC/20ºC (100µmols.m-2.s-1) and transferred to 5ºC. In two independent experiments, cold stress was applied after dawn or after dusk. Light intensity was reduced to 60-70µmols.m-2.s-1. After 0h, 1h, 2h, 8h, 12h, 16h and 24h of treatment, four plantlets were pooled and frozen in liquid nitrogen. Cold-acclimation experiments were conducted during ten days, also in 12h/12h (SD) or 16h/8h (LD) photoperiod. Plants were maintained during five days (D1-5) at 20ºC and then transferred to 12ºC. After five days of acclimation (D6-10), temperature was again reduced to 4ºC (D11). At D5, D6, D10 and D11, samples were collected and frozen in liquid nitrogen at 2h, 8h, 16h and 24h after dawn. ABA and dehydration treatments were performed by transferring plants to culture medium containing 100µM ABA and medium-free culture flasks, respectively. Mock controls were obtained by transferring the plants to new flasks containing micropropagation medium. After 10min, 30min, 1h, 2h, 8h and 12h four plantlets were pooled and frozen in liquid nitrogen.

2.9 Semi-quantitative RT-PCR

Two micrograms of total RNA were used for cDNA synthesis with SuperScript® First-Strand Synthesis System (Invitrogen) following manufacturers instructions. cDNA samples were diluted 1:3 in sterile miliQ H_2O and used as template for PCR with 1x GoTaq reaction buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.3 µM of each primer (Table 2) and 1U GoTaq® DNA Polymerase (Promega), in 20µL total volume. *PrdCBFs* were amplified using 2µL cDNA, while 1µL was used for the remaining genes. PCR was performed as follows: 5min incubation at 95ºC, followed by 24 to 32 cycles of 30sec at 95ºC, 30-40sec at corresponding annealing temperature (Table 2) and 40sec at 72ºC and a final extension step for 5min at 72ºC. *PrdActin* (AM491134) was used as internal control. Total reaction volumes were analyzed by electrophoresis on 1.2% agarose gel stained with ethidium bromide. Images were captured using the ChemiDoc XRS System (Bio-

Rad, Hercules, CA, USA). At least two technical replicates were performed for each gene.

Table 2. Primer sequences and corresponding amplification cycles and amplicon sizes for each primer combination used for semi-quantitative RT-PCR. F/R – forward/reverse primers; Anneal. – annealing temperatures; Ampl. – amplicon size.

Gene		Primer sequence	Anneal. (ºC)	# PCR cycles	Ampl. (bp)
PrdCBF1	F	5'-CGCTAATGAACAGGTTCTTCTCTCA-3'	58	32	550
	R	5'-TTCACACTATCCTTCTTCTTCTTCTTC-3'			
PrdCBF2	F	5'-CTCTAATGGACTTGTCTCAACTTTC-3'	58	32	540
	R	5'-CCAAGTTCACACTACCCTTCTTG-3'			
PrdDHN1	F	5'-CTTATGGTGGGGCTGGGTA-3'	62	23	453
	R	5'-TCGGTGGTCACAGACCTACA-3'			
PrdActin	F	5'-AGCAAGGTCCAGACGAAGAA-3'	58	24	385
	R	5'-TGTAGGTGATGAAGCCCAATC-3'			

3. Results

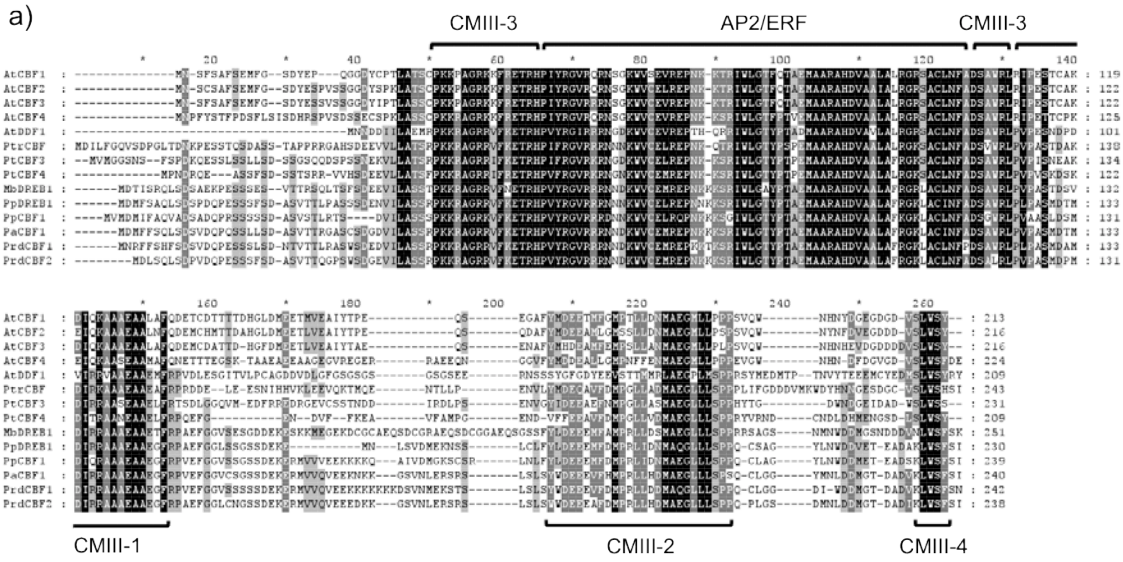
3.1 Isolation of two almond CBF*-like genes*

An almond CBF-like sequence (*PrdCBF1*) was initially isolated from cDNA samples obtained from cold treated *in vitro* almond plantlets, using a PCR-based approach with primers designed for the sweet cherry *PaCBF* (Kitashiba et al., 2004). Homology to CBF/DREB1 family of TFs was confirmed using BLASTX against *Genbank* database. Among the multiple clones sequenced during 3'RACE, an alternative 3' extension was obtained and, after 5'RACE, the identification of an additional member of the almond CBF gene family (*PrdCBF2*) was confirmed. *PrdCBF1* contained an open reading frame (ORF) of 729 nucleotides, encoding a 242 amino acid protein of 27.45 kDa with a pI 7.69. The deduced *PrdCBF2* ORF was slightly smaller than *PrdCBF1*, with 717 nucleotides, encoding a 238 amino acid protein with 26,59 kDa, pI 5.29. PrdCBF1 and -2 shared 82% amino acid identity. Pairwise sequence alignment within the *Prunoideae* CBFs already characterized revealed that PrdCBF1 and PrdCBF2 protein sequences are

closer to PaCBF1 than to peach PpCBF1 (Supplementary Table 2).

Multiple sequence alignment of PrdCBF1 and -2 predicted proteins with other CBFs from different species confirmed the conservation of the AP2 DNA-binding domain as well as conserved motifs CMIII-1 to -4 as previously defined by Nakano et al. (2006) (Figure 2a). These conserved motifs included the putative nuclear localization signal and the DSAWR signature (CMIII-3) flanking the AP2/ERF region. In phylogenetic analysis, the sequences belonging to the Prunoideae sub-family clustered together, demonstrating their high similarity (Figure 2b). Within this cluster, peach CBFs grouped separately from PrdCBF1, -2 and PaCBF1. The CBF/DREB1 peptide sequences from dicot species clustered in three major groups (Figure 2b, I, II and III). Interestingly, CBF-like proteins from the same species (*Betula pendula*, *Populus trichocarpa*) or genus (*Malus x domestica* and *Malus baccata*) showed up in two different groups (I and II) suggesting a different evolutionary origin. The *Prunus* CBFs isolated so far clustered in group II.

Since dehydrins are predicted targets of the CBF signalling pathway, the almond *Dehydrin1* (*PrdDHN1*) promoter and partial coding sequence was also isolated by PCR using specific primers targeting the reported sequence from peach, *PpDHN1* (AY819769; PPU62486) (data not shown). The *PrdDHN1* sequenced region was highly similar to the *PpDHN1*, sharing 97% nucleotide identity up to the initiation codon. The presence of two CRT/DRE elements was confirmed at -238 and -191bp from the deduced TATA-box, similarly to the *PpDHN1* promoter (Wisniewski et al. 2006).


a)
CMIII-3
AP2/ERF
CMIII-3
CMIII-1
CMIII-2
CMIII-4

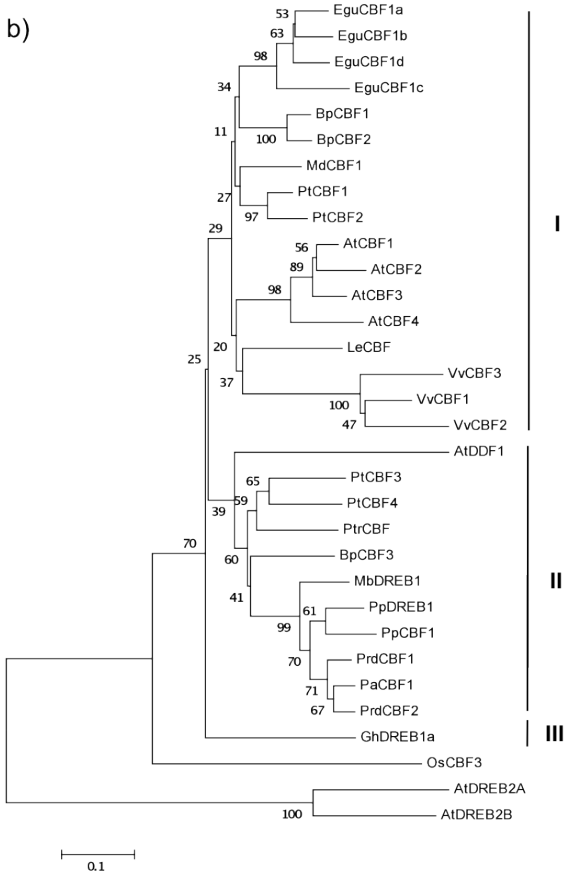

b)
EguCBF1a
EguCBF1b
EguCBF1d
EguCBF1c
BpCBF1
BpCBF2
MdCBF1
PtCBF1
PtCBF2
AtCBF1
AtCBF2
AtCBF3
AtCBF4
LeCBF
VvCBF3
VvCBF1
VvCBF2
AtDDF1
PtCBF3
PtCBF4
PtrCBF
BpCBF3
MbDREB1
PpDREB1
PpCBF1
PrdCBF1
PaCBF1
PrdCBF2
GhDREB1a
OsCBF3
AtDREB2A
AtDREB2B
I
II
III
0.1

Figure 2.

➤ **Figure 2.** (a) Multiple alignment of CBF amino acid sequences from *Prunus* spp. with other representative CBF sequences from *Arabidopsis* (AtCBF1-4; AtDDF1), poplar (PtCBF3-4), dwarf apple (MbDREB1) and citrus (PtrCBF), performed with ClustalW; (b) Phylogenetic relationships between CBF/DREB1 and DREB2 amino acid sequences from herbaceous and woody plant species. A consensus neighbor-joining tree was generated, based on multiple alignment, with bootstrap analysis with 1,000 replications. The sequences used for these analysis were: AtCBF1 (AY667247), AtCBF2 (AY667247), AtCBF3 (AY667247), AtCBF4 (NM_124578), AtDDF2 (NM_101131), AtDREB2A (NM_001036760), AtDREB2B (NM_111939) from *Arabidopsis thaliana*; BpCBF1 (EF530204), BpCBF2 (EF530205), BpCBF3 (EF530206) from *Betula pendula*; EguCBF1A (DQ241820), EguCBF1B (DQ241821), EguCBF1C (EU794855), EguCBF1D (EU794856) from *Eucalyptus gunnii*; GhDREB1A (AY321150) from *Gossypium hirsutum*; LeCBF1 (AY034473) from *Lycopersicon esculentum*; PtCBF1 (EF151456), PtCBF2 (EF151457), PtCBF3 (EF151458) from *Populus trichocarpa*, PtrCBF (DQ790889) from *Poncirus trifoliata;* VvCBF1 (AY390372), VvCBF2 (AY390376), VvCBF3 (AY390375) from *Vitis vinifera*; OsCBF3 (AF300970) from *Oryza sativa*; MdCBF1 (HM992942) from *Malus x domestica*; MbDREB1 (EF582842) from *Malus baccata*; PaDREB1 (AB121674) from *Prunus avium*; PpCBF1 (HM992943), PpDREB1 (EF635424) from *Prunus persica*; PrdCBF1 (JQ317157), PrdCBF2 (JQ317158) from *Prunus dulcis*. CBF/DREB1 sequences from dicotyledon species clustered in three groups: I, II and III. Bootstrap values are indicated on the branches. The bottom scale represents 0.1 amino acid substitutions per site.

3.2 PrdCBF1 *and* PrdCBF2 *promoter analysis*

To gain insight into the signalling mechanisms involved in *PrdCBF1* and *-2* transcription regulation we isolated their corresponding promoter regions by iPCR. Using *Nco*I and *Not*I digested genomic DNA as template, gene specific nested-PCR reactions generated one major band, for either *PrdCBF1* or *PrdCBF2* (Figure 3a and 3b, respectively), which was cloned and sequenced. This approach allowed the identification of sequences with 1401 bp and 1417 bp, upstream the deduced transcription start sites (TSSs) of *PrdCBF1* and *PrdCBF2*, respectively.

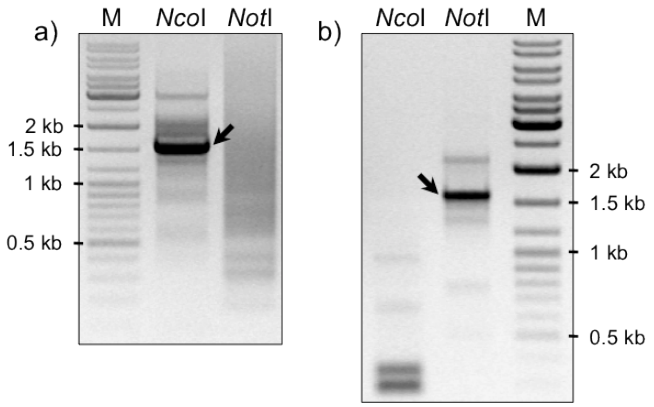


Figure 3. Nested-PCR amplification for iPCR using specific primers for *PrdCBF1* (a) and *PrdCBF2* (b). Almond genomic DNA was digested with *Nco*I or *Not*I and restriction fragments were self-ligated and used as templates for PCR-amplification using specific primers designed for known regions. To increase specificity, a nested-PCR was performed using a set of primers binding within the specific products from the first PCR. The fragments isolated for sequencing are indicated by black arrows. M – GeneRuler^{TM} DNA ladder mix (Fermentas).

The putative TATA-box motifs were located ca. 30 bp upstream the TSS (Figure 4a and 4b), close to two putative CAAT-box regions (not shown). *In silico* analyses of the -1000bp upstream TATA-box revealed the presence of several core sequence motifs related to environmental and endogenous signalling (Figure 4a and 4b). In particular, *PrdCBF1* and *PrdCBF2* promoters contained six to seven MYC motifs, which are putative binding sites for bHLH TFs, including ICE1 (Chinnusamy et al., 2003). Some of these MYC elements contained G-box core motifs specifically recognized by other bHLH TFs named Phytochrome-Interacting Factors (PIFs), which act in light/circadian clock-mediated transcription regulation (Martínez-García et al., 2000). The *PrdCBF1* promoter contained a conserved motif of ICEr2 close to TATA-box, which in *Arabidopsis* was found to be determinant for cold induction of *AtCBF2* (Zarka et al., 2003). In *PrdCBF2* promoter an ICEr2-like motif was also found in similar position. The *PrdCBF1* promoter contained at least six ABA-responsive elements (ABREs), while three were found in *PrdCBF2* promoter. Several MYB-related consensus motifs were

a)

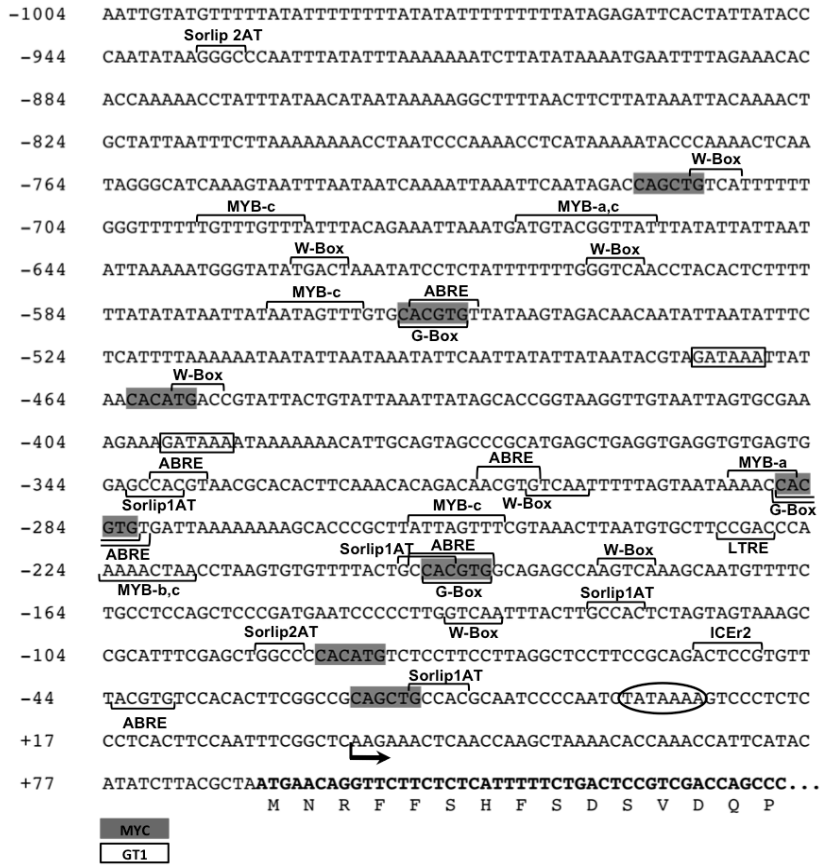


Figure 4. Analyses of the nucleotide sequence of the promoter regions of *PrdCBF1* (a) and *PrdCBF2* (b, next page), including the position of some predicted *cis*-elements. The sequences homologous to MYC and GT1 recognition sites are highlighted in grey and open boxes, respectively. The putative TATA boxes are indicated within circles. The arrows show the putative transcription initiation point predicted by 5'RACE.

also found in both promoters. These were related to several signalling pathways like osmotic stress (MYB-a) (Abe et al., 2003), defense (MYB-b) (Chakravarthy et al., 2003) and flavonoid biosynthesis (MYB-c) (Bodeau and Walbot, 1992; Solano et al., 1995). The presence of several W-Box regions also suggests that the WRKY family of TFs, generally involved in wounding, abiotic and biotic stress responses (Zhang and Wang, 2005), also regulates *PrdCBF*s. Finally, light-responsive elements such as GT1

(Zhou, 1999), SORLIPs (Hudson and Quail, 2003) and I-box (Terzaghi and Cashmore, 1995) were also found. Interestingly, *PrdCBF2* promoter contained substantially more GT1 and SORLIPs motifs than *PrdCBF1*, which suggests a putatively higher dependence on light regulation.

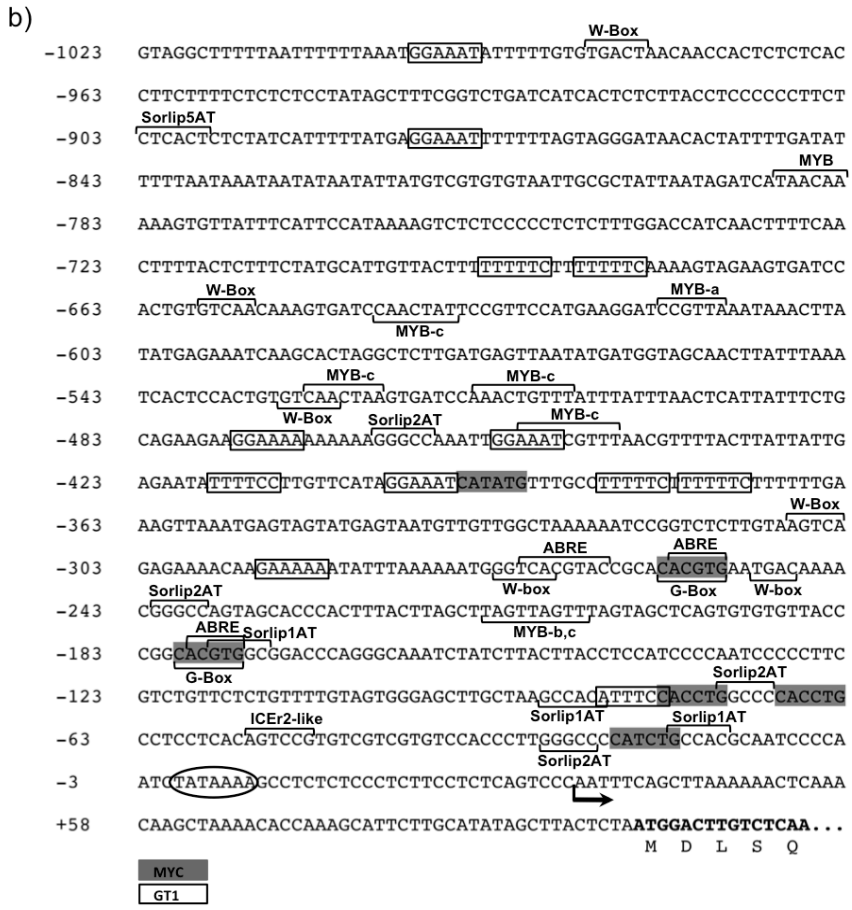


Figure 4. (*Continued*)

3.3 Mapping analysis of PrdCBF1 *and* PrdCBF2

The genetic mapping of *PrdCBF1, -2* and *PrdDHN1* was performed using the selective (bin) mapping analysis using the *Prunus* (almond x peach) reference map (Howad et al., 2005). CAPS markers for *PrdCBF1* and *PrdCBF2* amplicons were obtained using *Not*I and *Taq*I, respectively

(Figure 5a and 5b). *Hae*III also generated a polymorphic pattern for *PrdCBF1*, which was in agreement to that obtained for *Not*I (data not shown). After screening the set of 6 selected F2 individuals we observed that both genes shared the same genotypic patterns: A (5), H (12), H (23), H (30), H (34), A (83) (where A represents homozygous for 'Texas' and H the F1 heterozygous genotype). Comparison to the correspondent genotypes of the 67 defined bins (Supplementary Table 1) revealed that *PrdCBF1* and *PrdCBF2* co-localize within the bin 5:21 at linkage group (LG) 5. For *PrdDHN1*, the promoter fragment was used for mapping and a polymorphic recognition site for *Xba*I was found between both parental DNA sequences. This marker was screened in the 6 F2 plants resulting in the following genotypic pattern: H (5), H (12), B (23), B (30), A (34), B (83) (B represents homozygous genotype for Earlygold) (Figure 5c). This matched the pattern characterizing bin 7:41.

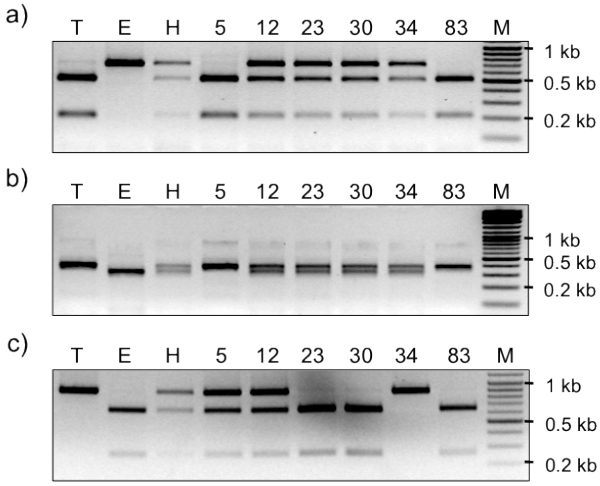


Figure 5: Bin mapping analysis of (a) *PrdCBF1*, (b) *PrdCBF2* and (c) *PrdDHN1* in almond x peach *Prunus* reference map. The coding regions of *PrdCBF1*, *-2* and the promoter region of PrdDHN1 were target for the development of polymorphic CAPS markers. Fragments were amplified from genomic DNA of parental cultivars ('Texas' [T] and 'Earlygold' [E]), F1 hybrid (H) and F2 selected plants (5, 12, 23, 30, 34, 83) and digested with *Not*I (a), *Taq*I (b) and *Xba*I (c). M – GeneRuler^{TM} DNA ladder mix (Fermentas).

Figure 6 shows the schematic representation of LGs 5 and 7 with the location of bins 5:21 and 7:41. Although with low precision, it was possible to observe that both bins overlapped the relative positions of two QTLs, previously found to influence blooming time and chilling requirements (Dirlewanger et al. 1999, Fan et al. 2010).

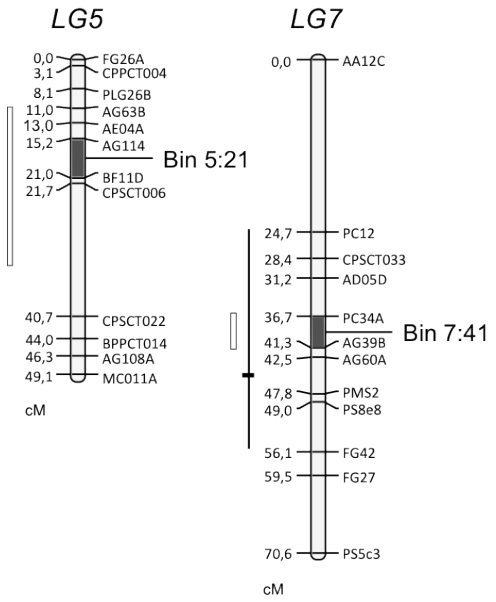


Figure 6. Predicted position of bins 5:21 and 7:41 (gray rectangles) and flanking molecular markers within linkage groups (LG) 5 and 7, as described by Howad et al. 2005. The approximate locations of QTLs related to blooming time are represented on the left side of LG5 and LG7 as: open vertical rectangles (Fan et al., 2010); thin vertical line (the thick horizontal line represents the site of the highest LOD) (Dirlewanger et al. 1999). cM – Centimorgan.

3.4 Genomic analysis of PrdCBF*s*

To investigate if additional genes similar to *PrdCBF1* and *-2* could be found in almond genome, we performed a Southern blotting hybridization using the complete *PrdCBF1* coding sequence as probe. Genomic DNA (cv. Verdeal) was digested with *Eco*RI, *Eco*RV and *Sst*I, which did not cut within the probe and hybridization was performed at medium stringency conditions (62ºC). This approach allowed the identification of at least eight to nine

bands in *Eco*RI lane, seven in *Eco*RV and four in *Sst*I (Figure 7). In the latter, the occurrence of four bands was confirmed in a replicate analysis (data not shown). To determine *PrdCBF1* and *-2* copy number in almond genome, DNA blots were hybridized to two labelled probes specific for *PrdCBF1* and *PrdCBF2*, respectively, also with no recognition sites for the selected enzymes. In both cases, hybridizations were carried out under high-stringency conditions (66ºC) and the hybridization pattern of each *PrdCBF* revealed the presence of at least two bands (Supplementary Figure 1). Considering that almond is highly heterozygous, due to self-incompatibility (Arús et al., 2009), this pattern may correspond to two putative allelic forms. Considering this, the occurrence of additional bands in the medium stringency blot (Figure 7) thus suggests that the almond *CBF* family may include 2 to 3 additional *CBF*-like members.

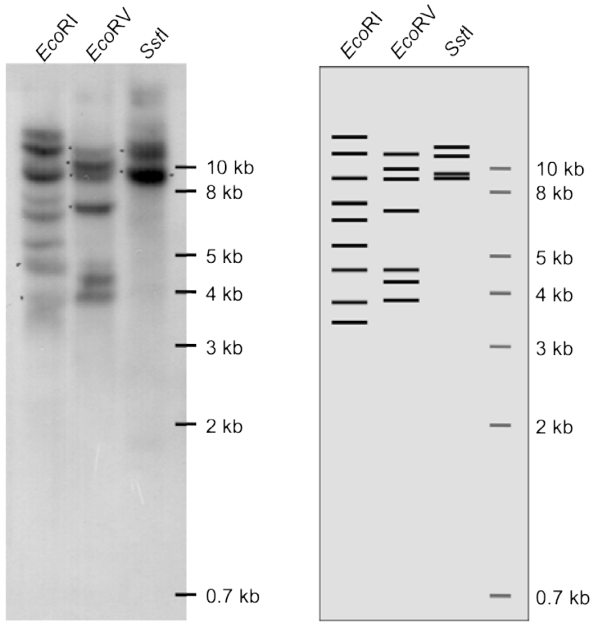


Figure 7. Southern blot analysis of almond genomic DNA. The *PrdCBF1* coding sequence was used as probe, in medium stringency conditions. The consensus pattern obtained from replicate analyses is represented on the right. The numbers on the right indicate the size of the fragments from ladder DNA (Fermentas).

3.5 Expression of PrdCBF1 *and* PrdCBF2 *is induced by low temperature*

As previously referred, *PrdCBF1* and *PrdCBF2* were isolated from cDNA samples obtained from cold-induced almond shoots submitted to 5ºC. To determine the expression pattern of both genes under low temperature treatment, we performed a gene expression analysis on *in vitro* almond shoots submitted to cold. Plants were cultured for 5 days at 12h/12h (SD) photoperiod and temperature decrease was imposed immediately after dawn. By semi-quantitative RT-PCR, we observed that *PrdCBF1* and *PrdCBF2* expression was induced at 2 and 1 hours, respectively, after temperature reduction (Figure 8a). *PrdCBF1* transcript accumulation increased along the day, reaching the highest level after 16h treatment (4h after dusk). For *PrdCBF2*, the results suggest that transcription induction may have started earlier than *PrdCBF1*, but showed a similar expression pattern along the 24h. Since the increase in transcript accumulation of both *PrdCBF* genes was observed towards the night period, we investigated if transcript induction could be positively regulated during the night period, by imposing cold stress immediately after the light period (Figure 8b). In these conditions we observed that both *PrdCBF1* and *PrdCBF2* expression reached a peak in expression at 8h after stress, declining to a steady state thereafter.

During light to dark cold treatment, *PrdDHN1* transcript levels showed to increase after 8h and throughout the night period (Figure 8a). However, in dark to light cold treatment, *PrdDHN1* transcript levels were already increased at 0h (Figure 8b), faintly decreasing after 2h of dark and increasing again at 8h and until the end of the treatment. Analysis of *PrdDHN1* expression at room temperature during the same period showed that this gene is actively transcribed at the end of day period, but decreasing during the night (Figure 8c). These results suggest that *PrdDHN1* transcription follows a circadian rhythm, which may be disrupted by low temperature.

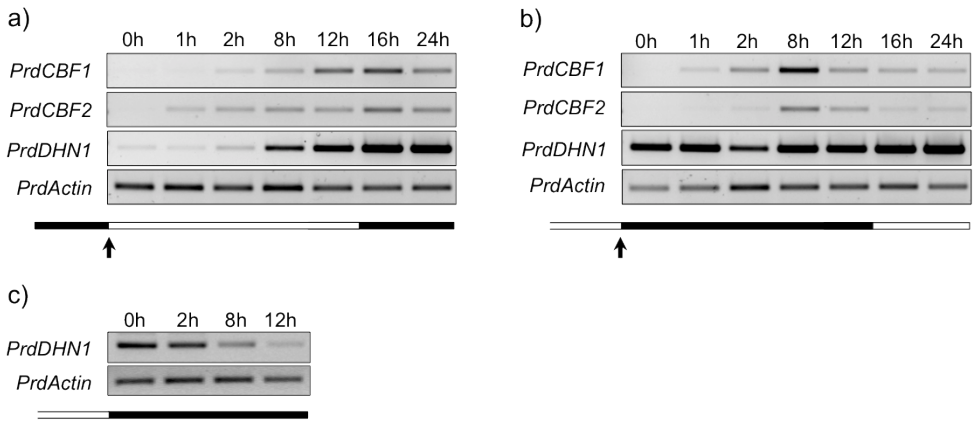


Figure 8. Analysis of *PrdCBF1, PrdCBF2* and *PrdDHN1* gene expression in almond *in vitro* shoots in response to cold induction treatment at 5ºC. *In vitro* plantlets were maintained SD photoperiod regime for 5 days under control growth temperatures. Cold (arrow) was imposed (a) after dawn or (b) after dusk. (c) Expression of *PrdDHN1* was also analyzed after dusk, under control conditions (20ºC). The white and black bars represent day and night period, respectively. Expression of *PrdActin* was used as control.

3.6 Expression of PrdCBF1 *and* PrdCBF2 *during cold acclimation*

Cold acclimation in perennial plants is regulated by a gradual reduction in temperatures together with the decrease in hours of light. To investigate whether both *PrdCBFs* could be differentially regulated during cold acclimation, we used *in vitro* almond shoots in a two-step cold acclimation treatment of five days at 12ºC, followed by 24h at 4ºC (Figure 9a), under two different photoperiods. Both *PrdCBF1* and *-2* were faintly expressed at room temperature (RT) during day 5 (D5, Figure 9b). Expression of *PrdDHN1* showed again a consistent induction at RT during the day period, decreasing after at least 16h at both photoperiods (D5, Figure 9b). This confirmed that light/dark cycling regulates *PrdDHN1* expression. When temperature decreased to 12ºC, expression of *PrdCBF1* and*PrdCBF2* was upregulated (D6, Figure 9b). In both photoperiod conditions, *PrdCBF1* transcript level was increased 2h after low temperature treatment, reaching a peak after 16h. *PrdCBF2* expression was increased

2h after cold induction when plants were kept in SD photoperiod (D6, Figure 9b), while under LD photoperiod, transcript level was upregulated only 8h after temperature decrease (D6, Figure 9b). In contrast to RT, *PrdDHN1* expression did not decrease 24h after exposure to 12ºC, reinforcing that the repressive effect on *PrdDHN1* transcription, occurring at RT before dawn, was prevented by cold. This may, at least in part, be related to the increase in PrdCBF1 and/or -2.

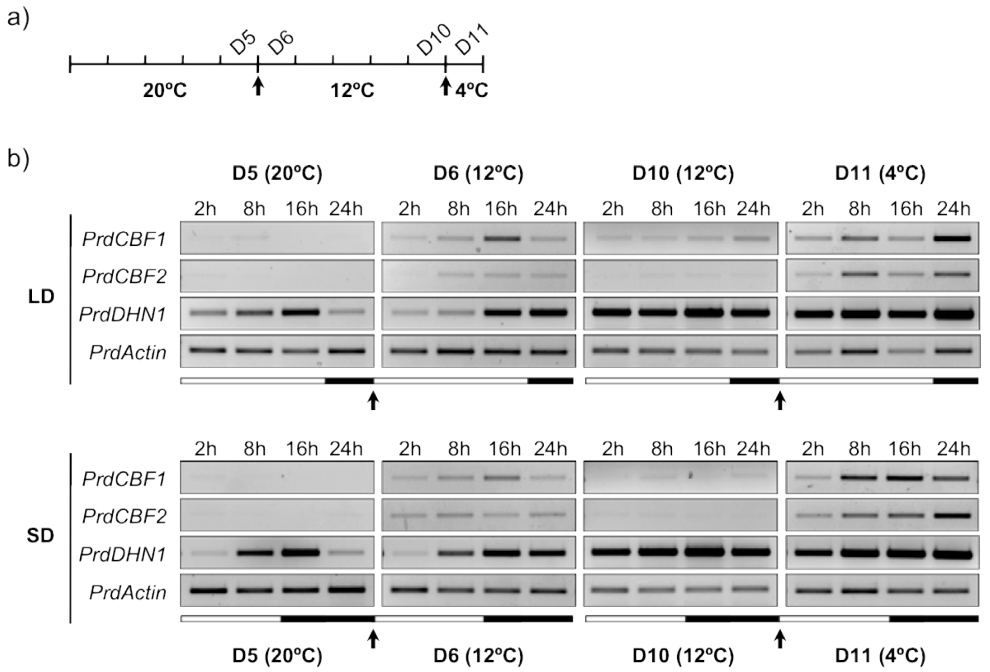


Figure 9. Expression analysis of *PrdCBF1, PrdCBF2* and *PrdDHN1* during a 10 days cold acclimation treatment depicted in (a), under LD or SD photoperiods (b). Almond *in vitro* plantlets were maintained in the corresponding photoperiod during 5 days (D1-D5), following acclimation to 12ºC during 5 days (D6-D10) and cold shock at 4ºC (D11) during 24h. The white and black bars below the pictures represent day and night period, respectively. Arrows represent the timing of temperature decrease. *PrdActin* expression was used as control.

After four days at 12ºC, transcripts of both *PrdCBFs* were still expressed but at a lower level as compared to D6 while *PrdDHN1* showed a

constitutive expression pattern (D10, Figure 9b). When temperature decreased to 4ºC (D11, Figure 9b) *PrdCBF1* and *-2* transcript level was again induced while no differences were observed in *PrdDHN1* transcription. Collectively, these results suggest that *PrdCBF1* and *PrdCBF2* are involved in cold acclimation, being induced when environmental temperatures decline to 12ºC, but keeping the responsiveness to lower temperatures.

3.7 PrdCBF1 *and* PrdCBF2 *are transiently induced by dehydration and ABA*

The effect of drought and ABA in *PrdCBF1* and *-2* expression was further investigated by transferring *in vitro* plantlets to dehydrating conditions (medium-free culture flasks) or to culture medium supplemented with 100µM ABA. In both conditions, *PrdCBF1* and *PrdCBF2* were slightly and transiently induced during the first hour after exposure, but transcript levels declined thereafter (Figure 10). The mock control showed a faint increase in transcript level, indicating that these genes could be also responding to mechanical stress, as also observed in other species (Gilmour et al. 1998). *PrdDHN1* transcript levels showed to increase throughout the 12h treatment in the three conditions (Figure 10). Nonetheless, during dehydration and ABA, transcript levels reached much higher levels, suggesting the involvement of this gene in the plant response to osmotic stress.

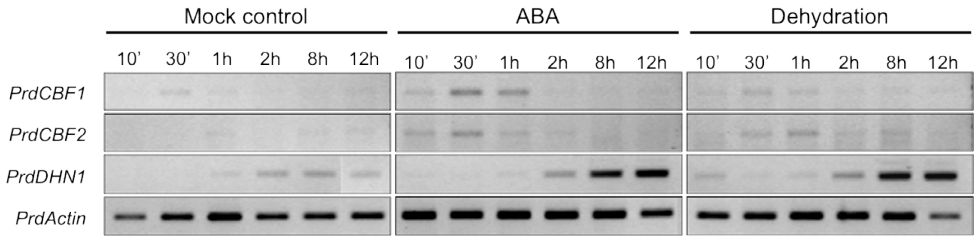


Figure 10. Analysis of *PrdCBF1*, *PrdCBF2* and *PrdDHN1* gene expression in response to 100µM ABA and dehydration. *In vitro* almond plantlets were transferred to solid culture medium supplemented with 100µM ABA or to medium-free flasks for dehydration. A mock control (mechanical stress) was also performed by transferring plants to new culture medium. *PrdActin* was used as control.

4. Discussion

In perennial fruit trees, temperature signals can modulate dormancy state and cold acclimation, which are determinant factors of winter survival. In Prunoideae, two *CBF* genes involved in cold acclimation were previously identified from sweet cherry (Kitashiba et al., 2004) and peach (Wisniewski et al., 2011) and mainly studied through heterologous expression. In the present work, we focused in almond, which is the earliest fruit tree to break dormancy but also shows the widest range of blooming dates among all the fruit and nut species (Socias i Company and Felipe, 1992).

PrdCBF1 and *PrdCBF2* genes were identified, sharing a high homology to *PaCBF1*, most probably related to the use of *PaCBF1* gene specific primers for cloning. Phylogenetic analysis showed that *Prunus* CBF sequences grouped within the same cluster, yet the two peach CBF sequences (PpCBF1 and PpDREB1) showed to be the most dissimilar. The phylogenetic tree obtained indicated that CBF/DREB1 proteins could segregate due to additional identity factors other than the speciation effect alone. In fact, *Prunus* CBFs grouped with poplar (PtCBF3 and -4), dwarf apple (MbDREB1), birch (BpCBF3) and *Arabidopsis* (AtDDF1) CBF members in cluster II, but other members from the same genus/species were also found in cluster I. In *Arabidopsis*, the *CBF* gene family is

composed of six members, which share common sequence structural features, but are induced by different signalling pathways: *AtCBF1* to *-3* (cluster I) are mainly involved in low temperature signalling (Shinwari et al., 1998, Gilmour et al., 1998); *AtCBF4* (cluster I) is expressed under drought and ABA treatments (Haake et al., 2002); *AtDDF1 and -2* (cluster II) are mainly related to salt response (Magome et al., 2004; Kang et al., 2011). Additionally, among the four poplar CBFs analyzed, expression of *PtCBF1 and -2* (cluster I) was induced by cold to a higher extent than *PtCBF3 and -4* (cluster II) (Benedict et al. 2006). Thus, although further studies need to be performed in this regard, the phylogenetic distribution obtained may relate the genes within each cluster to a common ancestor and/or functional aspect.

In *Arabidopsis* and tomato, three *CBF* genes are organized in a tandem array within chromosomes 4 and 3, respectively (Shinwari et al., 1998; Zhang et al., 2004). A similar tandem organization is also found for *CBF* genes from Poaceae (Tondelli et al., 2011). Mapping analysis in the almond x peach *Prunus* reference map revealed that *PrdCBF1* and *PrdCBF2* were co-localizing within a 5.8cM region in LG 5. In Southern blotting analysis, the two putative allelic forms of both *PrdCBF1* and *-2* were detected, in addition to other bands, demonstrating that the almond CBF family may be composed of at least four to five members. Considering that the three enzymes used did not generate the same number of bands, it is possible that the putative genes could be located within the same region, not being fully resolved by restriction. With the advent of the peach genome sequencing, available since April 2010 (International Peach Genome Initiative, http://www.rosaceae.org/peach/genome), gene identification in *Prunus* species has been facilitated. As determined for the almond genome by Southern blotting, the peach genome revealed the presence of 5 related sequences, located within the same genomic region (Supplementary Table 3; Supplementary Figure 2).

In silico analysis of *PrdCBF1* and *PrdCBF2* promoter regions predicted the location of several *cis*-elements putatively involved in transcriptional regulation. MYC elements are common motifs in CBFs promoters and in *Arabidopsis* these were shown to be recognition sites for the bHLH ICE1 TF. AtICE1 activates the expression of *AtCBF3* in response to low temperature, also regulating *AtCBF1* and *AtCBF2* cold induction but to a lower extent (Chinnusamy et al., 2003). In *AtCBF2* promoter, Zarka et al. (2003) identified a 125bp regulatory region determinant for cold-induced gene expression located close to the TATA-box (-33bp). It contained two sequence motifs, ICEr1 (MYC-type motif) and ICEr2 (ACTCCG), located -140bp and -44bp from the TATA-box, respectively. These showed to act in concert to control cold response, being themselves only weakly responsive (Zarka et al., 2003). Similarly, both *PrdCBFs* contained putative ICEr2 motifs, -48bp from TATA-box and several upstream MYC elements (within -120bp), which suggest that these regions may also be important to regulate *PrdCBF1 and -2* cold induction.

Gene expression analysis using *in vitro* propagated almond plantlets demonstrated that *PrdCBF1 and -2* are induced 1-2h after temperature decrease to either 5ºC or 12ºC. The expression pattern of both genes was similar and showed to depend on the time of the day at which temperature was imposed. When cold (5ºC) was applied during the night period, both *PrdCBFs* gene expression showed a consistent peak at 8h. However, when cold induction was applied during the light period, the upregulation was less pronounced reaching a peak at 16h, during the night period. The circadian clock and light have been shown to play a role in *CBF*s regulation. In *Arabidopsis*, cold-induced *CBF* transcription is gated by the circadian clock, showing rapid and higher expression induction when plants are transferred to cold during the light period (Fowler et al., 2005). In this case the possible effect of light/dark conditions was ruled out since the gene expression pattern was maintained under constant light conditions and was lost in

transgenic plants lacking circadian cycling (Fowler et al., 2005). Under control conditions, the expression of *AtCBF* genes also shows a circadian-regulated pattern, although in a lower extent than during cold, increasing during the light period and being repressed towards the night period (Franklin and Whitelam, 2007; Kidokoro et al., 2009). However, cold stress may abolish this circadian regulation in some extent (Bieniawska et al., 2008). Thus, if cold is applied during a period of permissive expression gated by circadian regulation, a rapid and higher response may occur. The expression pattern obtained for *PrdCBFs* suggests that, in this species, transcript induction is favoured at the end of the light period. Interestingly, this deduced pattern correlates to that of *PrdDHN1* observed at room temperature.

The induction of the *PrdDHN1* transcript level at room temperature, increasing towards the end of light period and decreasing during the night, was unexpected. Light-regulated *DHN* transcription and translation under cold or drought stress was previously reported in blueberry (Panta et al., 2001), wheat (Ohno et al., 2003) and sunflower (Cellier et al., 2000). Under control conditions, induction of *DHN* by light was only reported during de-etiolation in sunflower seedlings (Natali et al., 2007). However, transcription activation was not followed by subsequent increase in translation, since protein accumulation was not observed (Natali et al., 2007). Consequently, this cannot be ruled out for *PrdDHN1* and it needs to be confirmed in future analyses. The control of translation may be released only in case of stress perception, involving the activation of still unknown post-transcriptional factors (Natali et al. 2007). Daily cycling of *PrdDHN1* transcription may act as a preventive strategy against unpredicted cold spells. In our study transcript accumulation was found to occur towards the end of light period, which in nature also relates to thermoperiod transition. It would be interesting to analyze if the circadian clock plays a role in this regulation. Interestingly, cold treatment eliminated the *PrdDHN1* induction/repression

cycling, leading to a constant gene expression and thus showing the importance of the *PrdDHN1* to the cold responses.

During both cold acclimation experiments no significant variation in *PrdCBF1, -2* and *PrdDHN1* expression was found between photoperiods. It was previously reported that short-day (SD) and long-day (LD) photoperiods could differentially regulate the cold-induced expression of *CBF*s (El Kayal et al., 2006; Welling and Palva, 2008) as well as *DHN-like* genes (Fowler et al., 2001; Puhakainen et al., 2004; Welling et al., 2004). One-year old *Eucalyptus* plants grown under SD (8h light) during one week at 12ºC/8ºC (light/dark) showed an enhanced level of *CBF* expression during subsequent response to 4ºC, as compared to LD (16h light) (El Kayal et al., 2006). In three-months old birch seedlings, pre-exposure to SD (12h light) for one week followed by 4ºC treatment resulted in a significant increase in expression of a *DHN-like* gene, as compared to plants grown at LD (24h light) conditions (Puhakainen et al., 2004). Additionally, induction of birch *CBF* genes during 4ºC treatment, showed to be delayed in dormant tissues (induced for several weeks at SD), although some *CBFs* remained upregulated for a longer period than in LD-grown plants (Welling and Palva, 2008). However, in peach bark, expression of *PpDHN1* during cold acclimation was also not affect by photoperiod (Wisniewski et al., 2006). Altogether, these results reveal that SD effect on low-temperature response is complex and may vary between different species or growth conditions.

Physiological changes triggered during SD exposure, such as the adjustment of osmotic potencial (Welling and Palva, 2006), are associated with natural dormancy induction. These changes may cause extensive reprogramming of gene expression, leading, for example, to alterations in hormonal responses and down-regulation of genes involved in cell division, expansion and differentiation as well as in protein translation and turnover (Schrader et al., 2004; Bassett et al., 2006; Druart et al., 2007). Thus, a precise simulation of the complex network of environmental and

endogenous conditions regulating dormancy induction, to study the effect of SD on the regulation of *CBFs* may be hard to reproduce in laboratory conditions. *In vitro* cultured plants revealed to be a simple system suitable to demonstrate the *PrdCBF*s response to cold. However, the humidity contidions and the use of growth regulators in *in vitro* cultures may bias the response to stress conditions and affect cold hardiness (Baldwin et al. 1998). Thus, the use of pot grown trees in further analysis is advisable considering the complex physiological and molecular processes involved in cold acclimation and dormancy occurring during seasonal development.

ABA accumulates only transiently during cold stress and cold induced gene expression occurs mainly through an ABA-independent pathway, mediated by CBF TFs (Shinozaki and Yamaguchi-Shinozaki, 2000). Nevertheless, ABA and dehydration play an important role during or after dormancy induction in woody plants (Welling and Palva, 2006). When *in vitro* almond plantlets were subjected to ABA or dehydration, a transient induction of *PrdCBF1* and *-2* expression occurred after 10 min up to 1h. Similar results were obtained for other cold-induced *CBFs* from *Arabidopsis* (Knight et al., 2004) and grape (Xiao et al., 2006). ABA-responsive elements (ABRE) are present in both *PrdCBF*s promoters, as also reported in *CBF* genes from other species (Knight et al., 2004, El Kayal et al., 2006). Nevertheless, the functional role of ABRE motifs in *CBFs* promoters remains unknown. In fact, deletion of the ABRE motif present in the aforementioned 125bp *AtCBF2* promoter did not affect the induction of ABA-mediated transcription (Knight et al., 2004). W-box motifs, which are recognized by WRKY TFs and are present in promoter regions of genes involved in biotic and abiotic stress signalling (Rushton et al., 2011), were also predicted for the *PrdCBF1 and PrdCBF2* promoters. Increasing evidence points for a role of WRKY in the upstream regulation of ABA signalling (Rushton et al., 2011).

In the present work two *CBF* genes were identified in almond, *PrdCBF1* and *PrdCBF2*. These genes belong to a multigene family of at least 5 members, are responsive to low temperatures and modulated by light conditions. Although the role of light/circadian clock is still not fully understood, these genes are promising candidate players in the cold acclimation mechanism occurring during fall/winter development. Bin mapping analysis also predicted an association of *PrdCBF1-2* and *PrdDHN1* to QTLs regulating blooming time and chilling requirements (Dirlewanger et al. 1999, Fan et al. 2010) (Figure 6). Thus, further association studies, in addition to gene functional analysis, will be determinant to clarify their role in controlling these traits during seasonal development.

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7. Supplementary Data

Supplementary Table 1. Genotypes for the 6 selected F2 plants from 'Texas' (almond) x 'Earlygold' (peach) that characterize the 67 bins identified in the correspondent *Prunus* reference map (Howad et al., 2005).

T x E Plant [(a)]						BIN name	Start [(b)]	End [(b)]	I [(c)]
5	12	23	30	34	83				
B	H	B	H	H	H	1:14	AA08B	pchcms4	13.6
H	H	B	H	H	H	1:15	CC23	EPDCU5100	0
A	H	B	H	H	H	1:26	AG116A	AC47	10.0
A	H	B	H	H	B	1:28	PceGA59	AG29A	1.4
A	A	B	H	H	B	1:34	CPSCT036	PC85	5.6
A	A	B	H	B	B	1:50	MC044	TSA2	14.1
A	A	B	H	B	H	1:52	PST-P	BG08A	0
A	H	B	H	B	H	1:55	LY37	AB08B	1
A	B	B	H	B	H	1:73	*Lap-1*	FG8A	15.9
H	B	B	H	H	H	1:78	BF08A	*TubA3*	2.8
H	B	B	C	H	B	1:87	Em5B	AG36B	4.1
A	B	H	A	A	H	2:08	DB06Bd	AC10	8.1
A	H	H	A	A	H	2:13	UDP98-025	CPDCT044	2.9
H	H	H	A	A	H	2:20	*Pij1*	BPPCT004	4.7
H	H	A	A	A	H	2:25	BPPCT001	AG107A	4.1
B	H	A	A	A	H	2:26	FG220	PC5	0
B	H	A	A	H	H	2:28	AC27A	CPDCT004	0
B	H	A	A	H	B	2:34	AE03A	AD12A	2.4
B	H	A	H	H	B	2:38	pchgms1	AE07D	2.9
B	H	H	H	H	B	2:45	CPSCT021	CPSCT023	6.0
B	H	H	H	H	H	2:50	Omt1	CC125	2.7
B	H	H	H	H	B	3:04	BF06B	BC06A	4.0
B	H	B	H	H	B	3:06	BA05A	BA10A	0.8
H	H	B	H	H	B	3:12	AA04A	UDP97-403	4.3
H	H	B	A	H	B	3:14	STS834bA	AG7	0
H	H	B	A	H	H	3:22	AG32A	CC116	4.0
H	H	H	A	H	H	3:37	AB10A	UDP96-008	11.7
A	H	H	A	H	H	3:49	6Pgd-2	AF08B	11.3
H	H	H	H	H	H	4:18	BG05A	BPPCT040	18.4
H	H	A	H	H	H	4:22	new	new	0
H	H	A	H	B	H	4:27	pchgms5	CC3	2.8
H	A	A	H	B	H	4:28	AG62	BF07D	0
H	A	A	H	B	B	4:46	CPPCT003B	CPPCT024B	12.3
H	A	A	A	B	B	4:63	BPPCT036	BH02Ad	12.6
A	H	H	A	B	H	5:04	new	New	0.9
A	A	H	A	B	H	5:05	FG26A	FG26A	0
A	A	H	A	B	A	5:08	CPPCT004A	PLG26B	5.0
A	H	H	A	B	A	5:11	AF07A	AG63B	0
A	H	H	A	H	A	5:13	AG21B	AE04A	0
A	H	H	H	H	A	5:21	AG114	BF11D	5.8
A	H	H	H	A	A	5:41	CPSCT006	CPSCT022	19.0
A	H	H	B	A	H	5:46	BPPCT014	AG108A	2.3
A	H	B	B	A	H	5:49	AG33	MC011A	0

[(a)] The expected genotype of each F2 plant for each bin is represented by: A, homozygote for the female parent ('Texas'); B, homozygote for the male parent ('Earlygold'); H, heterozygote (F1).
[(b)] First ("Start") and last ("End") markers of the bin in the TxE reference map.
[(c)] Interval in cM between the first and the last marker of the bin.

➤ **Supplementary Table 1.** (*Continued*)

T x E Plant [a]						BIN name	Start [b]	End [b]	I [c]
5	12	23	30	34	83				
H	A	A	H	H	A	6:25	TSA4	PLG39D	20.8
H	A	A	B	H	A	6:39	BPPCT008	EPDCU2584	9.2
H	H	A	B	H	A	6:45	CPPCT023	pchcms5	3.2
H	H	A	B	H	H	6:49	AB06A	AA07A	0
B	H	A	B	H	H	6:56	FG4	BPPCT025	2.8
B	H	A	B	H	B	6:65	CPPCT047	PC60	6.4
B	H	A	B	B	B	6:74	UDP98-412	Pgl1	2.3
B	H	H	B	B	B	6:80	BC06B	Civ62A	0.8
B	B	H	B	B	B	6:84	CPPCT030	CPPCT021	3.5
A	H	H	B	H	B	7:25	AA12Cd	PC12	24.7
H	H	H	B	H	B	7:31	CPSCT033	AD05D	2.8
H	H	B	B	A	B	7:41	PC34A	AG39B	4.6
H	H	B	H	A	B	7:48	AG60A	PMS2	5.3
H	H	B	H	A	H	7:56	PS8e8	FG42	7.1
H	A	B	H	H	H	7:71	FG27	PS5c3	11.1
A	B	H	H	H	H	8:11	CPSCT018	BF08B	10.9
A	H	H	H	H	H	8:19	AG112A	CPPCT035	5.8
A	H	A	H	H	H	8:21	LY29	UDP96-019	0
A	H	A	H	H	A	8:23	EPDCU3516	CC131A	0
A	H	A	H	A	A	8:28	BPPCT012	AD01A	3.6
A	H	H	H	A	A	8:30	PC101	AB08A	0.8
H	H	H	H	A	A	8:41	AG4A	FG37	10.8
H	H	H	B	A	A	8:60	CPDCT023	PC36	17.1

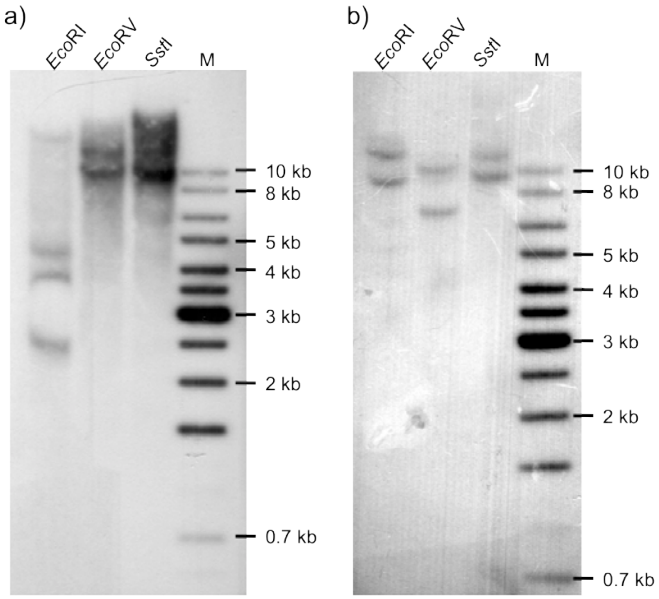
Supplementary Table 2. Identity scores (%) determined through pairwise alignment using ClustalW of PrdCBF1 and PrdCBF2 amino acid sequences in comparison to other *Prunus* CBFs and five *Arabidopsis* CBF members. Protein sequence length is indicated (aa – amino acid).

	PrdCBF1 (242aa)	**PrdCBF2** (238aa)
PaCBF1 (240aa)	85%	88%
PpDREB1 (230aa)	80%	77%
PpCBF1 (239aa)	73%	72%
AtCBF1 (213aa)	48%	49%
AtCBF2 (216aa)	47%	44%
AtCBF3 (216aa)	47%	47%
AtCBF4 (224aa)	49%	45%
AtDDF1 (209aa)	49%	51%

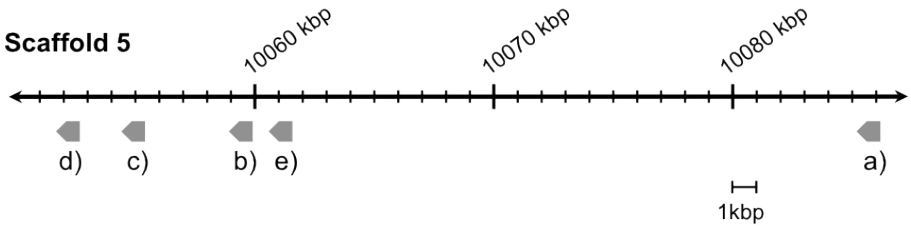
Supplementary Table 3. Predicted transcripts from the peach genome database (www.rosaceae.org/peach/genome) producing significant alignments with *PrdCBF1* full-length coding region using blastn program. The predicted positions of each transcript on scaffold 5 are mentioned in Supplementary Figure 2.

Predicted transcripts	Bit score	E-value	Position (Suppl. Figure 2)	Highest similarity *
ppa021197m	957	0.0	a)	*PrdCBF1*
ppa010800m	662	0.0	b)	*PrdCBF2*
ppa010909m	396	1e-110	c)	*PpDREB1*
ppa014628m	343	1e-93	d)	*PpCBF1*
ppa017761m	301	3e-81	e)	-
ppa010130m	44	0.001	-	-

(*) Individual pairwise alignments were generated for each peach or almond *CBF*s in order to search for the corresponding transcript in the peach genome.



Supplementary Figure 1. Southern blot analysis of *PrdCBF1* (a) and *PrdCBF2* (b) genes using the corresponding promoter regions as probes and high stringency hybridization conditions. M -GeneRuler™ DNA ladder mix (Fermentas).



Supplementary Figure 2. Schematic representation of a portion of scaffold 5 from peach genome, revealing the close proximity of five CBF-related genes, which showed high similarities to *PrdCBFs.* Letters a) to e) refer to Supplementary Table 3 (adapted from GBrowse, http://www.rosaceae.org/gb/gbrowse/prunus_persica/).

CHAPTER III

Functional Analysis of PrdCBF1 and PrdCBF2 in *Arabidopsis*

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Summary

PrdCBF1 and *PrdCBF2* are candidate players in the low temperature-signalling pathway in almond, being induced at the transcriptional level in response to temperature decline. To confirm whether both genes encode functional transcription factors (TFs), we analyzed their activity by heterologous expression in *Arabidopsis*. When transiently expressed in *Arabidopsis* protoplasts, both PrdCBFs were able to induce the expression of the *GUS* reporter gene driven by promoter regions harbouring CRT/DRE motifs, including the *PrdDehydrin1* (*PrdDHN1*) promoter. In addition, transgenic plants overexpressing PrdCBF2 showed a constitutive accumulation of endogenous members of the CBF regulon (*AtRD29A*, *AtCOR15A* and *AtXero2*) and also an increase in freezing tolerance, determined by electrolyte leakage assay. Most transgenic overexpressing lines showed dwarf phenotype and low seed set, which compromised the confirmation of these results for *PrdCBF1* and in several *PrdCBF2* lines. Nevertheless, the overall data gathered in this Chapter suggests that both *PrdCBFs* encode functional TF proteins.

1. Introduction

CBF transcription factors (TFs) play a fundamental role during cold acclimation in evolutionarily diverse plant species with contrasting levels of cold tolerance. The cold signalling pathway mediated by CBF/DREB1 TFs involve both upstream mechanisms, related to cold sensing and other regulatory factors that modulate the expression of CBF-encoding genes, and downstream components which include genes regulated in response to CBF activation. The deduced interplay of these components is determinant for plant response to low temperature, allowing cold acclimation and avoiding cellular damage. The 'downstream'-regulated genes are often referred as the CBF regulon. A global survey in *Arabidopsis* indicated that 306 genes, out of approx. 8000 genes, were cold responsive, and within this group, 12% were associated to CBF regulation (Fowler and Thomashow, 2002). Additional transcriptomic analysis during *Arabidopsis* cold response also evidenced the abundance of differentially regulated genes containing the CBF-specific CRT/DRE *cis*-elements, although redefining the global range of cold-responsive genes in the *Arabidopsis* genome (Kreps et al., 2002; Hannah et al., 2005; Vogel et al., 2005).

The CBF regulon covers a diverse group of functions, including cryoprotection (cold-responsive [COR], dehydrin [DHN] and other late embryogenesis abundant [LEA] genes), synthesis of proline and raffinose (compatible solutes also with cryoprotective activity), transcriptional regulation, sugar transport and protein fate, in addition to a number of putative or hypothetical proteins with unknown functions (Fowler and Thomashow, 2002). In fact, constitutive overexpression of CBFs in *Arabidopsis* activates this regulon in the absence of a cold stimulus, increasing freezing tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Fowler and Thomashow, 2002; Gilmour et al., 2004). The CRT/DRE element (consensus CCGAC) was first found in the promoter regions of

AtRD29A, *AtRD29B* (induced by dehydration, cold, salt or ABA) and *AtCOR15A* (cold-responsive) (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994). However, this sequence seems to be highly conserved among plants, including woody perennials, and is used to identify new CBF target genes in these species (Jiang et al., 1996; Puhakainen et al., 2004; Wisniewski et al., 2006; Wisniewski et al., 2011).

CBFs are members of a plant specific AP2 domain-containing protein superfamily, the AP2 (APETALA2)/EREBP (ethylene-responsive element binding protein) (Riechmann and Meyerowitz, 1998), but are distinguished by the presence of highly conserved CBF signature sequences including, PKKPAGR and DSAWR (Jaglo et al., 2001) (also named CMIII-3 [Nakano et al., 2006]), which flanks the AP2 domain. This domain has DNA-binding activity and the flanking regions are thought to impart specific targeting of CBFs to CRT/DRE sites. In fact, deletion or mutational analysis of PKKPAGR, the putative nuclear localization signal located upstream the AP2 domain, showed to affect *AtCBF1* binding ability, rather than its nuclear targeting (Canella et al., 2010). Furthermore, while the N-terminal regions containing these motifs relate to DNA recognition and binding, C-terminal regions (after DSAWR) are involved in transactivation of downstream target genes (Wang et al., 2005). Additional insights on the functional role of CBF TFs evidenced that the N-terminal regions may directly interact with members of chromatin-modifying coactivator protein complexes (Mao et al., 2006). Thus, CBFs appear to be involved in the recruitment of these complexes to CRT/DRE containing promoter regions, changing chromatin structure and allowing transcription initiation (Mao et al., 2006; Pavangadkar et al., 2010).

In Chapter II we reported the identification of two cold-responsive CBF genes in almond. These genes showed the basic structure expected for the CBF family of TFs, including the conserved motifs within the deduced N-terminal DNA-binding and C-terminal activation domains. Additionally,

these genes contained several *cis*-regulatory elements related to upstream regulation, also present in CBFs from other plants, and were transcriptionally induced during low-temperature stress. In the current chapter we aimed to study the downstream functional activity of *PrdCBF1* and *-2* encoding proteins, through transient expression in protoplasts and heterologous overexpression in *Arabidopsis*.

2. Materials and Methods

2.1 Biological material and growth conditions

The *Arabidopsis* mesophyl cell suspension line (ecotype Columbia) used for protoplast isolation, was provided by Dr. Laszlo Bogre and maintained as described by Mathur and Koncz (1998). Protoplast isolation was performed according to Anthony et al. (2004). Briefly, *Arabidopsis* cells (three to four days after sub-culture) were harvested by centrifugation and incubated in B5-GM media (1x Murashige & Skoog [MS] medium, including B5 vitamins [Duchefa Biochemie, Haarlem, The Netherlands]; 0.34M glucose; 0.34M mannitol; pH 5.5) supplemented with 1% (w/v) Cellulase and 0.2% Macerozyme enzymes (Duchefa Biochemie) for 3-4h in the dark. Protoplasts were carefully collected by centrifugation, washed in B5-GM and ressuspended again in B5-Sucrose medium (1x MS medium with B5 vitamins; 0.28M Sucrose; pH 5.5). After new centrifugation, protoplasts were recovered, quantified using a Fuchs-Rosenthal counting chamber and diluted in B5-sucrose medium up to a final concentration of $6x10^6$ protoplasts/mL.

Agrobacterium-mediated *A. thaliana* transformation was performed in ecotype Columbia by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain LBA4404. The binary vectors used contained the *bar* gene, which conferred plant resistance to Basta herbicide or to its active ingredient, phosphinothricin (PPT). Seeds were surface

sterilized by immersion in 50% (v/v) commercial bleach (4% sodium hypochlorite) and 1:1000 Tween-20 solution and washed six times in sterile distilled water. Seeds were then sown in MA medium (1x MS basal salt mixture; 0.5g/L MES buffer; 10g/L sucrose; 0.8% agar; pH 5.7) supplemented with 50mM PPT and 100mM Cefotaxime, and were stratified for three days at 4ºC in the dark. After stratification, selection of positive lines and subsequent screenings were performed according to Harrison et al. (2006).

2.2 Vectors for heterologous expression in Arabidopsis

Vectors for overexpression analysis were constructed using the Gateway® Technology system (Invitrogen, Carlsbad, CA, USA), according to manufacturers' instructions, unless otherwise stated. For transient expression and transactivation analysis in protoplasts two types of vectors were generated. Effector vectors were obtained by cloning *PrdCBF1* and *-2* coding sequences into pDONR221 and recombined with the pK7WG2 destination vector (Karimi et al., 2002), downstream the cauliflower mosaic virus (CaMV) 35S promoter. *PrdCBF1* and *PrdCBF2* coding sequences were amplified by PCR (using GwCBF1-F/R and GwCBF2-F/R, respectively [Table 1]) from cDNA samples obtained from *in vitro* almond plantlets exposed to cold stress (Chapter II), using Phusion® High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland). PCR was performed in 20µL volume as previously described (Chapter II). Reactions were incubated at 98ºC for 30sec, followed by 28 cycles of 10sec at 98ºC, 10sec at 60ºC and 20sec at 72ºC, with the final extension step at 72ºC for 5min. Reporter vectors were obtained by cloning the promoter regions of almond *Dehydrin1* (*PrdDHN1,* -770 to -126bp from ATG, accession n. JQ317156) and *A. thaliana RD29A* (-943bp to -23bp from ATG, accession n. At5g52310), through restriction-based cloning into pLUCm35GUS (Figueiredo et al., 2012). This vector contained the β-glucuronidase (GUS) reporter gene

driven by the minimal 35S (m35S), in addition to the Luciferase (LUC) gene under the control of CaMV35S (Figure 1a). Primers were designed to amplify the genomic regions containing the CRT/DRE motifs, upstream the predicted TATA-box. *Pst*I and *Sal*I recognition sites present right upstream the m35S promoter in pLUCm35GUS were included in the 5' end of each primer sequences. PCR was carried out as mentioned above, but using template genomic DNA from almond (Chapter II) and *A. thaliana* (DNeasy® Plant Mini Kit [Qiagen, Valencia, CA, USA]), respectively. Reactions were incubated for 30sec at 98ºC followed by 30 cycles of 10sec at 98ºC, 10sec at 58ºC and 30sec at 72ºC, with the final extension step for 5min at 72ºC. Amplified fragments were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). After restriction digestion with *Sal*I and *Pst*I (Fermentas Inc., Ontario, Canada) PCR fragments were ligated to the linearized pLUCm35GUS vector using T4 ligase (Invitrogen).

Table 1. Sequences of the primers used in vector construction for transactivation analysis and heterologous expression in *Arabidopsis*. Primer adaptor sequences used for Gateway (Gw) cloning are indicated in italic. Underlined sequences indicate the corresponding recognition sites for *Pst*I and *Sal*I used for restriction-based cloning.

Gene/Locus	Primer	Sequence
PrdCBF1	GwCBF1-F	5'-*GGGGACAAGTTTGTACAAAAAAGCAGGC* TCGCTAATGAACAGGTTCTTCTCTCA-3'
	GwCBF1-R	5'-*GGGGACCACTTTGTACAAGAAAGCTGGGT*CCT ACGTTGATTATTAATTGGAGAAACTC-3'
PrdCBF2	GwCBF2-F	5'-*GGGGACAAGTTTGTACAAAAAAGCAGGC* TCTCTAATGGACTTGTCTCAACTTTC-3'
	GwCBF2-R	5'-*GGGGACCACTTTGTACAAGAAAGCTGGGT*CCTA ATCGACTAAAATAAA CGTCGATGA-3'
prom*AtRD29A*	*Pst*I-RD29A-F	5'-GGCTGCAGGAGCCATAGATGCAATTC-3'
	*Sal*I-RD29A-R	5'-CCGTCGACAATAGAAGTAATCAAACC-3'
prom*PrdDHN1*	*Pst*I-DHN-F	5'-GGCTGCAGTAACATCAGATGAGACCC-3'
	*Sal*I-DHN-R	5' CCGTCGACGCAACGAATCTACACCCAAA-3'

For *Arabidopsis* genetic transformation, pDONR221 entry vectors containing *PrdCBF1* and *-2* coding sequences were recombined with pEarleyGate201 (Earley et al., 2006) destination vectors as described above. In these vectors, both coding regions were fused in frame with a N-terminal hemaglutinin (HA)-tag, under the control of the CaMV35S constitutive promoter.

2.3 Transactivation analysis

PEG-mediated transfection was performed according to Anthony et al. (2004) using 3:1 (6µg:2µg) equimolar ratios of effector:reporter vectors. Equal protoplast aliquots (50µL) were incubated for 10min with the corresponding vector combinations, prior to addition of 150µL polyethylene glycol (PEG) solution (25% [w/v] PEG 6000; 0.45M mannitol; 0.1M $Ca[NO_3]_2$). Reactions were incubated for 10 min in the dark, and PEG was removed by centrifugation after serial dilutions with 0.275M $Ca(NO_3)_2$ solution. Protoplasts were then ressuspended in B5-GM medium and incubated in the dark at room temperature. Three replicate transfections were prepared for each vector combination and control. Twelve hours after transfection, protoplasts were lysed using CCLR buffer (100mM Potassium Phosphate, 1mM EDTA, 7mM 2-mercaptoethanol, 1% [v/v] Triton, 10% Glycerol) and cell debris was removed by centrifugation. GUS activity was assessed by monitoring cleavage of the β-glucuronidase substrate 4-methylumbelliferyl β-D-glucuronide (MUG [Duchefa Biochemie]) (Jefferson, 1987). Protein extracts (20µL) were mixed with 0.5µL MUG 50mM and incubated for 1h at 37ºC. Reaction was stopped with 180µL Na_2CO_3 200mM. Fluorescence was measured on a FLUO-star plate reader (BMG Labtechnologies Inc., Durham, NC, USA) at 455nm when excited at 365nm. As internal transfection control, the same volume of each protein extract was assayed for LUC activity using a Modulus™ Microplate luminometer (Turner Biosystems) with dual injectors. Extracts were mixed with 150µL

LUC assay reagent (10mM Tricine pH7.8, 5mM $MgCl_2$, 0.1mM EDTA, 3.3mM DTT and 2mM ATP) and 0.5µM Luciferin (Biotium Inc., Hayward, CA, USA), and luminescence was measured with 10sec integration time. GUS and LUC values were calculated as the average of three independent readings. GUS/LUC ratios were calculated and results are shown as the average for each transfection replicates.

2.4 Gene expression analysis in transgenic plants

Transgenic (T2) lines overexpressing *PrdCBF1* or *-2* (HA-PrdCBF1/2) and Columbia wild-type (WT) were grown in MA medium and maintained in a growth chamber 16h/8h photoperiod at 22ºC/20ºC (light/dark). Twenty days after germination (DAG) temperature was decreased to 0ºC at the beginning of the light period and samples were collected 6h after cold exposure and frozen in liquid nitrogen.

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturers' instructions, treated with DNase I (Ambion® Turbo DNA-*free*™ Kit, Applied Biosystems, Carlsbad, CA, USA) and quantified using Nanodrop (Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was performed using 2µg of total RNA, with Transcriptor High-Fidelity cDNA synthesis kit (Roche Diagnostics) following manufacturers' instructions. cDNA samples were diluted 1:3 in sterile H_2O and 1µL was used as template for PCR with 1U GoTaq® DNA Polymerase (Promega Corp., Madison, WI, USA), 1x GoTaq reaction buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.4µL of each primer, in 20µL total volume. Two microliters of cDNA were used for expression analysis of *AtGA2OX3*. PCR was performed as follows: 5min incubation at 95ºC, followed by 24 to 32 cycles of 30sec at 95ºC, 30-40sec at corresponding annealing temperature (Table 2) and 40sec at 72ºC, and a final extension step for 5min at 72ºC. *Arabidopsis Actin1* and α*-Tubulin* genes were used as housekeeping

genes. PCR reactions were analyzed as previously mentioned in Chapter II. At least two technical replicates were performed for each gene.

Table 2. Sequences and corresponding amplification conditions and amplicon size (Ampl.) for the primer combinations used for semi-quantitative RT-PCR. F/R, forward/reverse primers; Anneal., annealing temperatures.

Gene (Accession n.)		Primer sequence	Anneal. (ºC)	# PCR cycles	Ampl. (bp)
PrdCBF1 (JQ317157)	F	5'-CGCTAATGAACAGGTTCTTCTCTCA-3'	56ºC	30	550
	R	5'-TTCACACTATCCTTCTTCTTCTTCTTC-3'			
PrdCBF2 (JQ317158)	F	5'-CTCTAATGGACTTGTCTCAACTTTC-3'	56ºC	30	540
	R	5'-CCAAGTTCACACTACCCTTCTTG-3'			
AtCBF1 (AT4G25490)	F	5'-CTGGACATGGAGGAGACGT-3'	58ºC	26	320
	R	5'-TTTTCCACTCGTTTCTACAACAA-3'			
AtRD29A (AT5G52310)	F	5'-GAACACTCCGGTCTCTCTGC-3'	56ºC	24	511
	R	5'-TGATGGAGAATTCGTGTCCA-3'			
AtXero2 (AT3G50970)	F	5'-CACCAGAATCAAACCGGAGT-3'	56ºC	26	572
	R	5'-TAGTGATGACCACCGGGAAG-3'			
AtCOR15A (AT2G42540)	F	5'-GGCCACAAAGAAAGCTTCAG-3'	56ºC	26	401
	R	5'-AATGTGACGGTGACTGTGGA-3'			
AtGA20OX1 (AT4G25420)	F	5'-GAGCCGCTTCTTTGATATGC-3'	58ºC	30	445
	R	5'-ATGGTCTTGGTGAAGGATGG-3'			
AtGA2OX3 (AT2G34555)	F	5'-ACCGACTCAGATGCCAAAAC-3'	58ºC	32	468
	R	5'-CTTCTCCGGGTAATGGTTCA-3'			
AtActin1 (AT2G37620)	F	5'-GGCGATCAAGCTCAATCCAAACG-3'	58ºC	26	391
	R	5'-GGTCACGACCAGCAAGATCAAGACG-3'			
Atα-Tubulin (AT4G14960)	F	5'-ATTGAGCGACCCACCTACAC-3'	56ºC	26	428
	R	5'-GTGGGTGGCTGGTAGTTGAT-3'			

2.5 Protein extraction and Western blotting

For protein extraction, 100mg of grounded plant material was homogenized with protein extraction buffer (50mM Tris-HCl pH 8.0; 150mM;

2mM EDTA pH8.0; 0.4% [v/v] Triton X-100; 2x Complete protease inhibitor cocktail [Roche]) and protein extracts were recovered after centrifugation and stored at -80ºC. Quantification was performed using the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) with BSA as the standard. Equal amounts of total protein extracts (100 µg) were separated by SDS-PAGE (5% stacking and 12% resolving gel) and transferred to a polyvinylidene fluoride (PVDF) membrane (PerkinElmer, Waltham, MA, USA) by electrotransfer. Membranes were blocked for 1h at room temperature, in a solution of Tris-buffered saline 1x (TBS) and 5% non-fat dry milk, and then incubated overnight (4ºC) with an anti-HA primary antibody (Mouse Monoclonal Anti-HA-Alkaline Phosphatase [F-7], Sigma-Aldrich) at 1:1000 dilution. The use of this specific antibody dispensed the incubation with a secondary antibody, so membranes were washed with 1x TBS and detection was performed using Western Lightning Plus ECL detection solution (PerkinElmer) and exposed to an Amersham Hyperfilm™ ECL (GE Healthcare, Uppsala, Sweden). Coomassie Brilliant Blue staining was performed for loading control.

2.6 Electrolyte leakage

The electrolyte leakage test was performed according to Verslues et al. (2006). Transgenic and WT plants were grown on MA medium as mentioned above for 15 days and then transferred to soil and grown for further 15 days. Two to three leaves were collected (in case of dwarf transgenic lines, full rosette leaves were used) and placed in sterile falcon tubes containing 100µl sterile water. Samples were placed in a growth chamber (Fitoclima D1200-PLH, Aralab, Portugal) and freezing temperatures were applied by gradual temperature decrease, -1ºC each 30min. When temperatures reached -1ºC, the tubes were opened and leaves were sprinkled with a small amount of ice chips, to allow ice nucleation. Samples (three replicates) were removed from the chamber at

0ºC, -4ºC and -8ºC and allowed to thaw on ice during 12h. Ten milliliters of sterile water was then added to the tubes and leaves were maintained in solution for another 24h. Solution conductivity was measured using a pH/EC/TDS/Temperature Meter (Hanna Instruments, Woonsocket, RI, USA). The total ion composition present in each sample was determined by incubating the tubes at 100ºC during 1h and measuring total conductivity. The percentage of electrolyte leakage for a given temperature was given by the ratio between the conductivities before and after 100ºC incubation.

3. Results

3.1 Transient expression of PrdCBF1 and PrdCBF2 in Arabidopsis protoplasts

The CBF family of TFs recognizes conserved CRT/DRE response elements found in the promoters of *COR* genes, activating their expression. To test whether PrdCBF1 and PrdCBF2 are functional TFs, their transactivation activity was evaluated through transient expression in *Arabidopsis* protoplasts. Vectors containing the *AtRD29A* or *PrdDHN1* promoter regions (promAtRD29A or promPrdDHN1, respectively) cloned upstream the m35S::GUS cassette were used as reporters. promPrdDHN1 contains two copies of the CRT/DRE motif in its promoter region, being a predicted PrdCBF target (Chapter II). *AtRD29A* is a widely known stress responsive gene, which is a target for CBF TFs in *Arabidopsis*, and the corresponding promoter contains four copies of the CRT/DRE *cis*-element. As a control to normalize transfection efficiency, reporter vectors also contained the 35S::*LUC* cassette (Figure 1a). Protoplasts were cotransfected with the effector vectors carrying 35S::*PrdCBF1* or 35S::*PrdCBF2* together with each reporter vector. Basal GUS/LUC ratios calculated for promAtRD29A-m35S::*GUS* or promPrdDHN1-m35S::*GUS*, with no effector were higher than that of the empty reporter vector

(m35S::GUS, Figure 1b). Since both *AtRD29A* and *PrdDHN1* are involved in stress response, this result might be related to the stress conditions imposed to protoplasts during PEG-mediated transformation. In the presence of either PrdCBF1 or PrdCBF2, GUS/LUC ratios were upregulated >2-fold as compared to the controls without effector. Additionally GUS/LUC ratios also increased in the protoplasts cotransfected with m35S::GUS and either one of the effectors. A detailed DNA sequence analysis of the original vector backbone revealed the presence of a CRT/DRE-like motif, -180bp upstream the m35S, which may act as a binding site for CBF TFs, promoting expression of reporter gene even without the presence of a stress-related promoter. The insertion site of promPrdDHN1 and promAtRD29A was located -13bp from m35S, moving that 'endogenous' motif far upstream in promAtRD29A-m35S::*GUS* and promPrdDHN1-m35S::*GUS* reporter vectors. Collectively, these results suggest that, *in vivo*, PrdCBF1 and PrdCBF2 are functional transactivator proteins.

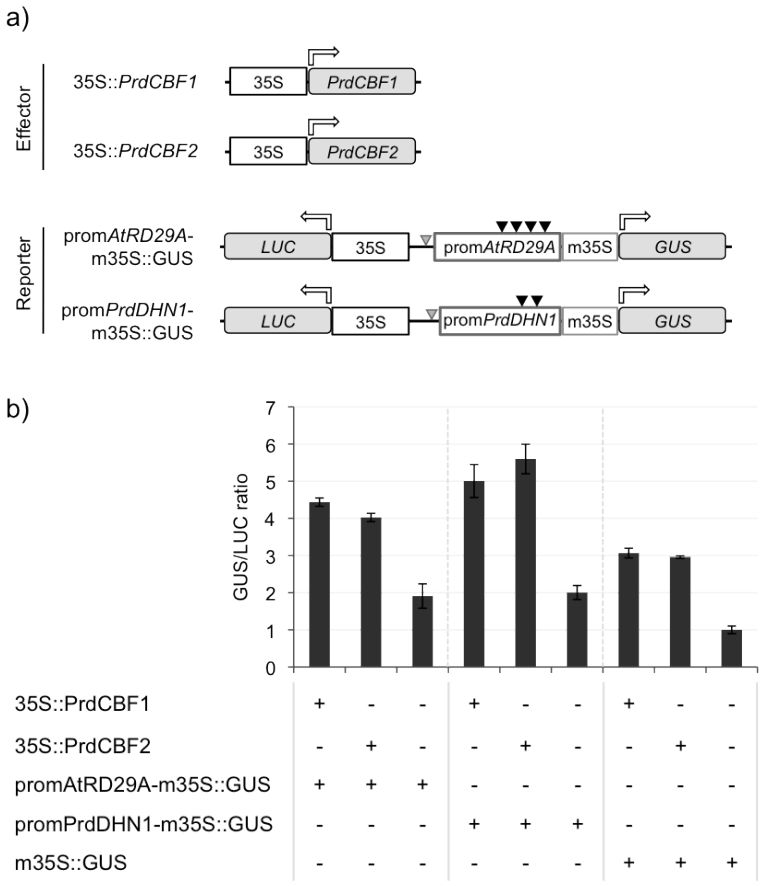

Figure 1. Analysis of PrdCBF1 and PrdCBF2 transactivation activity. (a) Schematic representation of the effector and reporter constructs used for transient expression in *Arabidopsis* protoplasts. Effector vectors contained the *PrdCBF1* and *-2* coding sequences driven by the constitutive 35S promoter. In the reporter vectors, expression of the *β-glucuronidase* (GUS) reporter gene was driven by the promoter regions of *PrdDHN1* or *AtRD29A* fused to the minimal 35S (m35S). For transfection control, these vectors also contained the *Luciferase* (LUC) gene fused to the 35S promoter. Arrows indicate transcription. Black arrowheads indicate the CRT/DRE *cis*-elements from promPrdDHN1/promAtRD29A; gray arrowheads indicate a CRT/DRE-like present in the reporter vector backbone. (b) Transactivation of stress-regulated promoters from *PrdDHN1* and *AtRD29A* genes fused to m35S, driven the expression of the GUS reporter gene. Each reporter vector was transformed alone or in combination with the *PrdCBF1* or *-2* containing effector vectors. GUS activity was measured and normalized for the constitutive LUC activity values. The indicated GUS/LUC ratios are means of three independent transformation reactions (± standard deviation), which were normalized for m35S::GUS.

3.2 Heterologous overexpression of PrdCBF1 and PrdCBF2 in Arabidopsis

In order to demonstrate the functional activity of PrdCBF1 and -2 *in planta*, *Arabidopsis* plants were transformed with vectors carrying a 35S::HA-PrdCBF1/2 cassette (Figure 2a). Unfortunately, we were unable to efficiently obtain or propagate transgenic lines, given the high number of false positives or T1 plants showing dwarf phenotype and hampered development obtained for both constructs. A total of five transgenic T1 lines showed dwarf phenotype (two for HA-PrdCBF1 and three for HA-PrdCBF2), and most of these did not produce sufficient amount of viable seeds to allow further analyzes, or died before reaching reproductive stage (data not shown). Therefore, only one transgenic line efficiently expressing the transgene was obtained for each construct, HA-PrdCBF1.1 and HA-PrdCBF2.4 (Figures 2b). The progeny of these T1 plants showed a 3:1 segregation ratio for PPT resistance, implying that the transgene was likely integrated at one locus. HA-PrdCBF1.1 phenotype was similar to WT plants while HA-PrdCBF2.4 showed dwarf and delayed development (Supplementary Figure 1). This line also showed defective anthesis, since the majority of flower buds remained closed (Supplementary Figure 2) but, unlike other T1 with similar phenotype, this line was able generate viable seeds. By Western-blot analysis, using an anti-HA antibody targeting the fusion protein, no protein accumulation was observed in HA-PrdCBF1.1 T2 plants, whereas HA-PrdCBF2.4 plants were efficiently expressing the HA-PrdCBF2 fusion protein (Figure 2c). Similar levels of accumulation were observed in plants grown under control growth conditions and cold (6h at 0ºC).

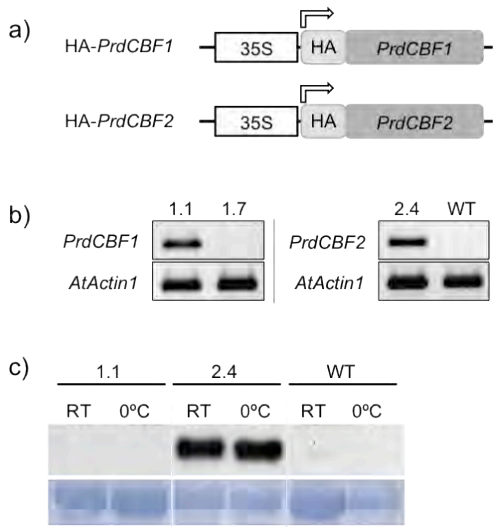

Figure 2. Heterologous expression of PrdCBF1 and PrdCBF2 in *Arabidopsis*. (a) Schematic representation of the constructs used for heterologous overexpression of PrdCBF1 and -2. *PrdCBF1* and *PrdCBF2* coding sequences were 5' fused to the hemaglutinin (HA)-tag, downstream the 35S promoter. (b) Transcript accumulation of *PrdCBF1* and *PrdCBF2* in HA-PrdCBF1.1 (1.1) and HA-PrdCBF2.4 (2.4) T2 plants (20 DAG), respectively, grown under control conditions (22ºC). HA-PrdCBF1.7 line (1.7, false-positive for PrdCBF1 overexpression) and wild type (WT) are shown as negative controls. *AtActin1* was used as internal control. (c) Western-blot using an anti-HA antibody for protein extracts obtained from HA-PrdCBF1.1 (1.1), HA-PrdCBF2.4 (2.4) T2 plants and WT (20 DAG), grown under control growth conditions (RT, 22ºC) or exposed to 0ºC during 6h. Coomassie Blue staining is shown below, as loading control.

To investigate if overexpression of HA-PrdCBF2 would lead to the activation of *COR*/stress-responsive genes, transcript accumulation of predicted targets of endogenous AtCBFs was analyzed. In WT plants, expression of *AtCOR15A*, *AtXERO2* and *AtRD29A* along with *AtCBF1* was induced after 6h of exposure to 0ºC (Figure 3a). Under control conditions these genes were only faintly expressed, or at least at a lower level than at 0ºC, in WT. In turn, HA-PrdCBF2.4 T2 plants showed increase expression of the endogenous CBF-targets, with no obvious modification of *AtCBF1* (Figure 3a). In HA-PrdCBF1.1, the expression pattern of these genes was similar to WT, in agreement to the undetected levels of fusion protein previously mentioned. Thus, almond CBF2 appears to be a functional TF in

Arabidopsis, in agreement with the results observed previously by transient expression in protoplasts. The absence of translation products resulting from transgene expression in HA-PrdCBF1.1 did not allow drawing the same conclusions for PrdCBF1.

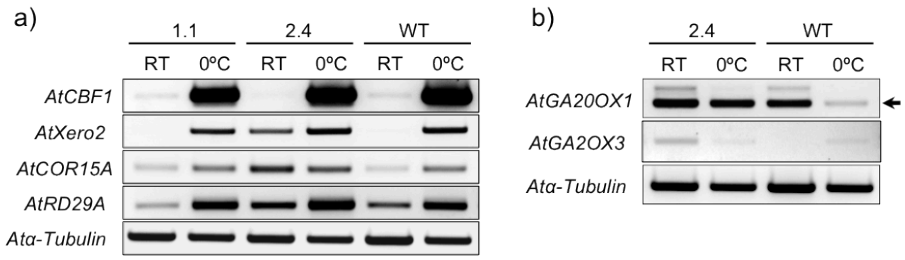

Figure 3. Transcript levels of genes involved in (a) cold acclimation and (b) GA metabolism in HA-PrdCBF1.1 (1.1), HA-PrdCBF2.4 (2.4) T2 and WT plants (20 DAG) grown under control conditions (RT, 22ºC) or after 6h exposure to 0ºC (as described in 2.3 section from Materials and Methods). In (b) the arrow indicates the fragment of interest for *AtGA20OX1*. Expression of *Atα-Tubulin* gene was analysed as internal control. Number of PCR cycles used for semi-quantitative RT-PCR are mentioned in Table 2.

Considering that overexpression of CBF genes may affect growth through changes in the gibberellin (GA) biosynthetic pathway (Achard et al., 2008), we further assessed the effect of HA-PrdCBF2.4 accumulation on the expression of two genes related to this pathway, *AtGA20OX1* and *AtGA2OX3* (Figure 3b). Expression of *AtGA20OX1*, which is related to GA biosynthesis, was detected in WT at RT conditions, but showed to be downregulated after 6h at 0ºC. In HA-PrdCBF2.4 plants, transcript accumulation in control conditions was similar to WT, but in cold conditions it did not decrease as observed in WT. In turn, expression of *AtGA2OX3* was nearly undetected in WT, while in transgenic plants it showed to be induced, particularly in control conditions. These results suggest that overexpression of PrdCBF2 affects the GA signalling pathway, at least in the line studied.

3.3 Freezing tolerance in transgenic plants

An electrolyte leakage assay was performed to evaluate the effect of the HA-PrdCBF2 overexpression on plant freezing tolerance (Figure 4). HA-PrdCBF1.1 line was also tested and, in agreement to the undetected changes on COR gene expression under RT, no significant difference was observed between HA-PrdCBF1.1 and WT. Conversely, HA-PrdCBF2.4 plants showed improved freezing tolerance since at -4ºC these plants lost, on average, about 30% of total electrolytes, while WT and HA-PrdCBF1.1 showed approximately 70% ion leakage. At -8ºC no significant differences were observed.

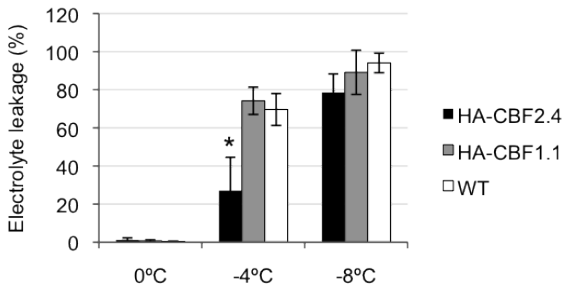

Figure 4. Electrolyte leakage (mean ± SD [n=3]) measured in leafs from non-acclimated HA-PrdCBF1.1, HA-PrdCBF2.4 T2 plants and WT (30 DAG), after exposure to 0ºC, -4ºC and -8ºC during 30 minutes. * Significant difference ($p<0.05$) to WT and HA-PrdCBF1.1, determined by Student's *t* Test. These results are representative of two independent experiments.

4. Discussion

CBF proteins from several plant species have been shown to bind to CRT/DRE *cis*-elements and regulate the expression of their target genes (Jaglo-Ottosen et al., 1998; Champ et al., 2007; Xiao et al., 2006; Dubouzet et al., 2003; Qin et al., 2004). As previously reported in Chapter II, genes encoding PrdCBF1 and -2 were induced during low temperature response, being putatively involved in cold acclimation mechanisms in almond. The functional activity of these putative TFs was further tested through transient expression in *Arabidopsis* protoplasts. This showed to induce GUS expression driven by CRT/DRE containing sequences from promPrdDHN1

or promAtRD29A. These results suggested that both *PrdCBF1* and *-2* genes encode functional TF proteins. A similar effect was observed when effector vectors were cotransfected with the control reporter vector, containing only the m35S::GUS cassette and not the specific target promoter. However, the cotransfection of another effector construct, expressing a non-CBF TF, with the same reporter vector did not affect GUS expression (Figueiredo, 2012). Therefore, we suggest that the presence of a CRT/DRE-like motif, harbouring the CCGAC consensus sequence, upstream the m35S::GUS cassette may have caused induction of GUS expression. Nevertheless, these results suggest that PrdCBF1 and -2 proteins act as transcriptional activators of CRT/DRE containing sequences.

Overexpression of CBF TFs in model plants such as *Arabidopsis*, rice (*Oryza sativa*) or tobacco (*Nicotiana* spp.) has been extensively used as a tool for gene functional analysis. This approach generally leads to an increase in abiotic stress tolerance under freezing, salt and drought conditions (Kasuga et al., 1999; Gilmour et al., 2000; Dubouzet et al., 2003; Gilmour et al., 2004; Kitashiba et al., 2004; Ito et al., 2006; Shan et al., 2007; Gutha and Reddy, 2008; Welling and Palva, 2008; Huang et al., 2009). However, the transgenic lines reported in these studies also evidenced dwarf and delayed development under normal growth conditions, and in some cases low seed set. In fact, the degree of growth retardation observed in transgenic *Arabidopsis* lines overexpressing AtCBF3 or OsCBF3 correlated with the level of transgene expression and degree of stress tolerance (Liu et al., 1998; Dubouzet et al., 2003). Additionally, recent reports demonstrated that CBF TFs may also regulate developmental processes such as vegetative growth and senescence, in addition to freezing tolerance (Achard et al., 2008; Sharabi-Schwager et al., 2010), implying that the CBF regulation should be fine tuned depending on the developmental context. One of the explanations for these effects on growth and development is related to the effect on GA metabolism. Achard et al.

(2008) reported that the overexpression of *AtCBF1* in *Arabidopsis* hampers growth by reducing the endogenous levels of GA, which in turn allows the accumulation of DELLA proteins involved in growth repression (and which degradation is stimulated by GA). This regulation seems to be included in an integrative response of plants during cold acclimation resulting in a limitation of plant growth, since cold or *AtCBF1* overexpression also affects the expression of genes related to GA catabolism (Achard et al., 2008).

In the present study we intended to perform similar functional study through heterologous overexpression of HA-PrdCBF1 and -2 in *Arabidopsis*. However, in addition to the low transformation efficiency observed, the occurrence of retarded growth and development led to the some difficulties in the maintenance and propagation of transgenic lines. In fact, from five transgenic T1 lines showing a dwarf phenotype, only HA-PrdCBF2.4 was able to produce sufficient (but still limiting) amount of viable T2 seeds to allow a preliminary characterization. Although we were able to obtain a homozygous T4 progeny, these plants showed a phenotype similar to WT and reduced expression of the transgene and the corresponding protein product (Supplementary Figure 3). Considering that the transgene expression was recovered after treatment with the hypomethylating agent 5-azacytidine (Supplementary Figure 3), we concluded that transcription repression of the transgene in homozygous plants was related to increased DNA methylation, most likely at the transgene locus. The occurrence of this epigenetic mark has been widely associated with transcriptional or post-transcriptional (PTGS) gene silencing, usually occurring in straight correlation with the transgene copy number integrated in the genome (Vaucheret and Fagard, 2001). More specifically, PTGS has been observed in homozygous plants for the transgene locus or related to complex transgene arrangements, being more efficiently triggered when strong constitutive promoters are used (Vaucheret et al., 2001; De Buck et al., 2001), which is the case of CaMV35S. PTGS results in the specific

degradation of transgene RNAs, mediated by homologous small interfering RNAs, and DNA methylation has been associated with a PTGS maintenance mechanism throughout development. Thus, PTGS may also explain the lack of detection of HA-PrdCBF1 protein in HA-PrdCBF1.1 T2 plants, although *PrdCBF1* transcription was detected.

In this scenario, we have decided to perform a preliminary characterization of PrdCBF2, using HA-PrdCBF2.4 T2 plants, bearing in mind the limitations related to the use of a single transgenic line. Heterozygous T2 plants were efficiently expressing the transcript and the corresponding translation product, and this showed to have an effect on the expression of endogenous CBF targets. In fact, expression of *AtCOR15A*, *AtRD29A* and *AtXERO2* was high in HA-PrdCBF2.4 grown under control temperature, while in WT, similar expression levels were only detected after cold-treatment. More particularly, *AtXERO2* was analyzed not only because it belongs to the AtCBFs regulon, but also because it is the closest *Arabidopsis* homolog to the peach *DHN1* (Bassett et al., 2009) and *PrdDHN1*. This may indicate *PrdDHN1* as a direct target of PrdCBF2 in almond. In HA-PrdCBF2.4 homozygous T4 plants, expression of COR genes showed a similar pattern to WT (data not shown) in agreement to the reduced accumulation of HA-PrdCBF2 protein product. Furthermore, overexpression of PrdCBF2 also led to changes in the expression of genes related to GA biosynthetic pathway, which may have justified the observed growth retardation. The increased expression of *AtGA20OX1* and *AtGA2OX3* in HA-PrdCBF2.4 lines was in agreement with the results previously obtained for the overexpression of *AtCBF1* (Achard et al., 2008). Achard et al. (2008) proposed that the increase in *GA 20-oxidase* genes expression correlated to a feedback loop mechanism induced in response to a decrease in endogenous GAs and accumulation of DELLA proteins. In the case of *GA 2-oxidase* genes, which are involved in GA catabolism, the increase in expression was also observed in WT in response to cold

(Achard et al., 2008). These authors proposed that low temperature and/or constitutive *AtCBF1* expression reduces bioactive GA levels through the upregulation of *GA 2-oxidase* genes (via a still unknown indirect pathway).

The increase in freezing tolerance deduced from the electrolyte leakage test may be related to the activation of *COR* gene expression in HA-PrdCBF2.4 line under normal growth conditions. These results suggest that PrdCBF2 is able to activate members of the endogenous CBF regulon in *Arabidopsis*. This led to a major decrease in plasma membrane damage under freezing conditions without previous acclimation to cold temperatures, as compared to WT plants. Overexpression of the sweet cherry PaCBF1 (sharing 88% amino acid similarity with PrdCBF2) in *Arabidopsis* led to increased survival under freezing temperatures and also under high salt concentrations. Preliminary root growth assays in salt-supplemented medium (85mM NaCl) using HA-PrdCBF2.4 T2 plants did not show significant differences as compared to WT (data not shown), although survival rates under higher NaCl concentrations were not assessed.

In addition to growth retardation, HA-PrdCBF2.4 T1 and T2 plants also showed defective anthesis (Supplementary Figure 2), which affected fertilization and seed set. In these plants (and additional T1 lines not producing viable seeds) the majority of the flower buds remained closed. Although this phenotype has never been reported in CBF overexpressing plants, a direct link between PrdCBF2 and flower development is difficult to establish given the limitations of having only one transgenic line to study. However, in the scope of this work, a putative (negative) effect of PrdCBF2 in flower development (and also in GA metabolism) should be considered, in the context of winter dormancy in almond. Considering that flower development in this species occurs during winter, it would be interesting to analyze in more detail the role of PrdCBF2 in floral organ development, dormancy break and anthesis. In this case, the choice of a suitable working model would be of extreme importance, as well as the use of more

appropriate promoters for heterologous expression, which could impose moderate changes in the development of transgenic plants.

5. Acknowledgements

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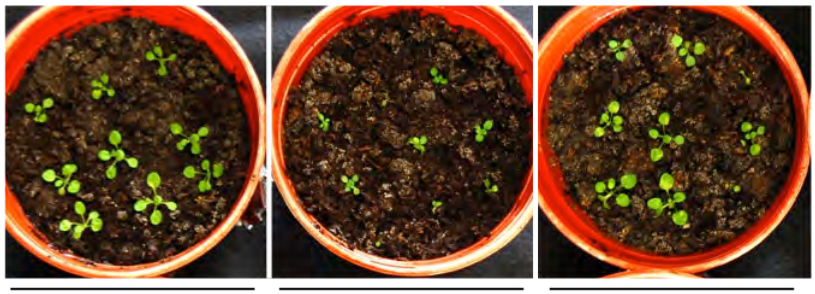
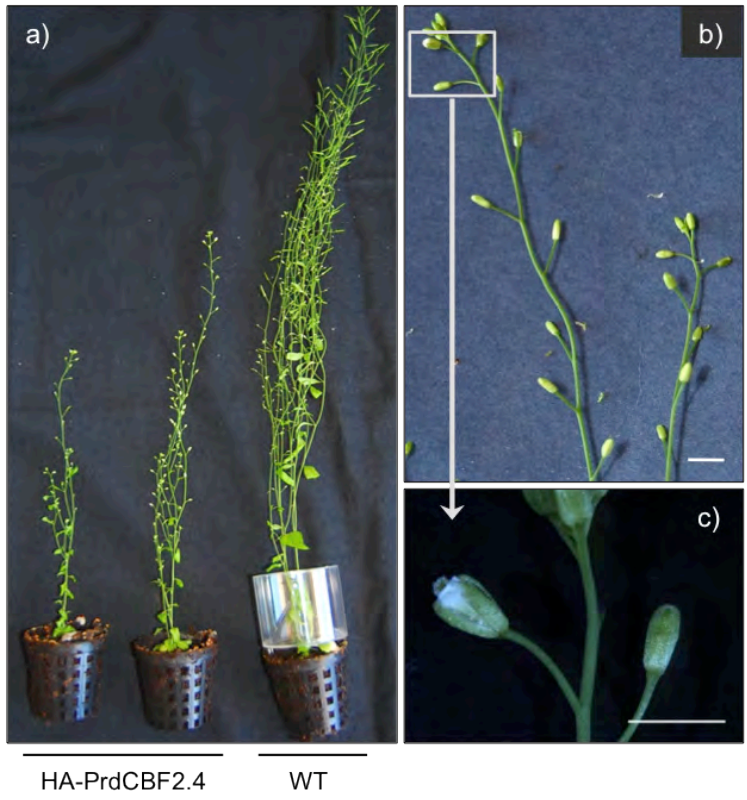
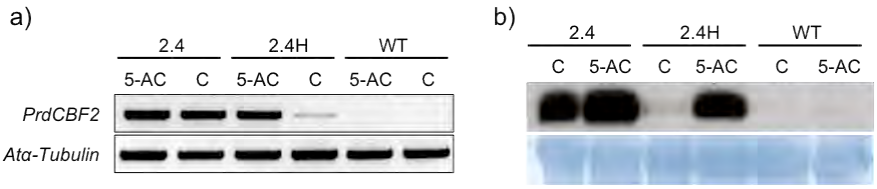
7. Supplementary Data



Supplementary Figure 1. Growth phenotypes of HA-PrdCBF1.1 and HA-PrdCBF2.4 T2 plants in comparison to WT plants (20 DAG).



Supplementary Figure 2. (a) Comparison between two representative HA-PrdCBF2.4 T2 plants and a WT plant, 75 DAG. Besides retarded growth most of HA-PrdCBF2.4 flower buds showed defective anthesis, since the majority of the flower buds remained closed (b) or showed only partial anthesis (c), with no expansion of the corolla. Scale bar represents 5mm.



Supplementary Figure 3. HA-PrdCBF2 transgene expression detected for HA-PrdCBF2.4 line in T2 heterozygous (2.4) and T4 homozygous (2.4H) plants. Transgenic and WT plants were grown for four days in culture MA medium, and then transferred to liquid MA medium (C) or liquid MA supplemented with 50 µM 5-aza-2'-deoxycytidine (5-AC, Sigma-Aldrich, USA). After eight days, plants were once again transferred to regular solid MA and allowed to grow for three additional days prior harvesting. RNA and protein extraction, cDNA synthesis, semi-quantitative RT-PCR (a) and Western-blot (b) were performed as mentioned in Materials and Methods. Homozygous plants showed low accumulation of the HA-PrdCBF2 transcription (a) and translation (b) products, but the treatment with 5-AC reverted this repression to levels similar to T2 heterozygous plants. Expression of *Atα-Tubulin* gene was used as internal control for RT-PCR and Coomassie Blue staining is represented as loading control for Western-blot.

CHAPTER IV

PrdCBF1 and *PrdCBF2* are Downregulated After Bud Break in Almond Trees

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Summary

In temperate fruit trees, seasonal dormancy and cold acclimation have a major impact on fruit production. Dormancy break is associated with chilling temperatures perceived in dormant stages and further promotive temperatures in spring. To gain insight into the still unclear molecular processes underlying blooming, the expression of genes putatively involved in cold response was studied in almond, which is the earliest Rosaceae fruit tree to bloom. Transcript levels of two *C-repeat Binding Factor* genes (*PrdCBFs*) and one of their putative targets *PrdDehydrin1* (*DHN1*) were analysed in flower buds and shoot internodes along seasonal dormancy up to bud break. In parallel, the expression of candidate genes related to flower development was also followed. In a two-year-long study, *PrdCBF2* showed a progressive increase in transcript abundance during fall in close correlation with cold acclimation while high transcript levels of PrdCBF1 and *PrdDHN1* were found already since summer. After bud break, with temperatures still within the chilling range, both *PrdCBFs* and *PrdDHN1* were found to sharply reduce transcription in flower buds and internodes, suggesting damping of CBF-mediated cold-signalling during growth resumption, in correlation with cold hardiness decline. Flower bud break was also followed by a decrease in the expression of *PrdGA20OX*, a candidate gene involved in gibberellin biosynthesis, and an increase in the expression of two homeotic genes related to floral organ development (*PrdMADS1* and *-3*). These genes may also be relevant players in the regulation of anthesis in this model Rosaceae species.

1. Introduction

Perennial trees are able to induce dormancy of actively growing tissues in order to cope with the more extreme environmental conditions occurring during fall and winter. The correct timing for dormancy induction and release is critical in plant adaptation and survival in the perennial habit (Tanino et al., 2010). After an active growth period in summer, perception of short-day (SD) photoperiods is one of the first environmental cues for bud set, growth cessation and dormancy (Nitsch, 1957). Conversely, dormancy release and growth reactivation in spring is induced after sufficient exposure to chilling temperatures (Perry, 1971). Fall/winter dormancy may be divided in two distinct, successive stages. Endodormancy is induced in early fall and is controlled by endogenous factors, up to the fulfilment of specific chilling requirements. Subsequently, ecodormancy starts when growth ability is regained, although bud break will only occur after the exposure to promotive warm temperatures, during late-winter/spring.

Dormancy induction and maintenance in woody plants involve extensive reprogramming of transcriptional and metabolic pathways (Schrader et al., 2004; Bassett et al., 2006; Renaut et al., 2008; Druart et al., 2007; Karlberg et al., 2010) in addition to morphological and physiological changes in meristems and surrounding tissues, which prevent cell growth and proliferation (van der Schoot and Rinne, 2011). Modulation of gibberellic acid (GA) metabolism has been also correlated to SD-induced seasonal dormancy. In poplar, cellular GA levels were shown to be rapidly downregulated during SD (Olsen et al., 1997) being related to growth cessation (Eriksson et al., 2000). Moreover, induction of abiotic stress-regulated genes was also observed in response to SD, prior to cold exposure, in trees such as poplar (Druart et al., 2007; Rohde et al., 2007; Ruttink et al., 2007), Norway spruce (Asante et al., 2011) and peach (Basset et al., 2006). This response may be explained by a decrease in

water content and osmotic potential detected during dormancy induction (Rinne et al., 1998; Welling et al., 2002; 2004).

After dormancy induction, the decrease in average temperatures in fall leads to an increase in cold hardiness and freezing tolerance, which is thought to reach maximum levels during winter (Rinne et al., 1998). Low temperature signalling pathways mediated by the CBF/DREB1 family of transcription factors (TFs) are obvious candidate players in this process. The major insight into the role of CBF TFs during seasonal development in woody plants came from studies in controlled environment during short-term exposure to cold stress (e.g. Benedict et al., 2006; El Kayal et al., 2006; Welling and Palva 2008; Navarro et al., 2009; Wisniewski et al., 2011; Chapter II). In these studies, *CBF* genes were rapidly induced after temperature decrease, with transcript accumulation generally peaking after 2h, and decreasing to a lower level after 24h of exposure to constant temperature. Additionally, transcriptional induction of *CBFs* by cold stress showed to be differentially regulated by photoperiod (El Kayal et al., 2006, Welling and Palva, 2008) or between tissues (Benedict et al., 2006). Considering that, within the same species, different *CBFs* showed different levels and patterns of accumulation (Benedict et al., 2006; Welling and Palva 2008; Navarro et al., 2009; Wisniewski et al., 2011) it is easy to predict that the role of each *CBF* could be slightly different during specific developmental stages. However, little is known about the role of specific *CBFs* during fall and winter, in response to the fluctuating environmental conditions to which field-grown trees are exposed. A major contribution to understand the role of low temperature signalling pathways came from the study of *cold-regulated* (*COR*) genes, which play a fundamental role in stress tolerance and are deduced targets for CBF TFs. More particularly, *Dehydrin*-like (*DHNs*) transcripts or corresponding protein products were shown to accumulate during endodormancy or in response to chilling (e.g. Artlip et al., 1997; Welling et al., 2004; Wisniewski et al., 2006; Yamane et

al., 2006). In poplar cambial meristems, transcription induction of *COR* genes, during cold-hardiness development in fall could be grouped according to specific timing of induction (Druart et al., 2007). This supported the hypothesis of a stepwise development of cold hardiness, sequentially induced by SD and temperature decrease (Druart et al., 2007).

As previously mentioned, growth ability is achieved when chilling requirements are met, while subsequent bud break is limited by low temperatures and promoted by warm temperatures. Recent evidences indicated that GA biosynthetic genes are induced by long-term chilling exposure in dormant buds, being associated with the acquisition of growth ability (Druart et al., 2007; Karlberg et al., 2010; Rinne et al., 2011). In poplar vegetative buds, GAs are further involved in a growth-promotive pathway by restoring the symplastic transport and signalling, which was previously blocked during dormancy induction (Rinne et al., 2011). In addition, dormancy-activity transitions may affect the acclimation state observed in dormant buds, since growth resumption is also often associated with a decrease in cold hardiness levels, in part associated with tissue/cellular rehydration (Kalberger et al., 2006). Differential regulation of stress-related genes has been also observed after prolonged exposure to chilling temperatures (Karlberg et al., 2010). Consistent with this, expression of specific *DHN* genes also appears to be downregulated in buds during dormancy break (Welling et al., 2004; Yamane et al., 2006; Yakovlev et al., 2008; Garcia-Bañuelos et al., 2009).

Although the regulation of cold acclimation and dormancy in perennial plants has received increased attention over the last years, the role of the molecular pathways involved in low temperature signalling is still poorly understood. In Rosaceae fruit species, as well as in other temperate fruit trees, flower initiation occurs the year before blooming and organogenesis inside flower buds is arrested during fall/winter dormancy. The timing of anthesis, as well as the maturity of reproductive organs at this

stage, may be critical factors influencing fertilization and fruit production in this species (Alburquerque et al., 2002; Ruiz et al., 2010; Campoy et al., 2011). Almond is the earliest fruit tree to bloom in winter/spring, but it also shows a wide adaptability to different environments (Alonso et al., 2010; Socias i Company and Felipe, 1992). In this work, the role of two almond *CBFs* was investigated along natural fall/winter dormancy period in adult almond trees selected for their early blooming. The expression pattern of four candidate genes involved in organ identity and GA metabolism was also studied with a focus on floral development prior to anthesis. Gene expression analysis performed in shoots and flower buds in two consecutive years showed that *PrdCBF1*, *-2* and *PrdDHN1* are active during the cold acclimation process, reducing expression after bud break. In flower buds, this response occurs in parallel with the induction of floral homeotic genes and a putative modification in the GA biosynthetic pathway. The role of temperature variation and deacclimation in gene expression during ecodormancy is further discussed.

2. Materials and Methods

2.1 Plant material

Seasonal gene expression studies were performed using three adult almond trees growing in the Lisbon area (Monsanto forest park, lat. 38º 43' 28.34" N, long. -9º 11' 35.55" W, Portugal). Sample collection was performed from late summer (September) to mid-winter (February), at approximately 15 days intervals, from 9am to 11am, during two consecutive years – 2009/10 (year-I) and 2010/11 (year-II). Developing flower buds and one-year-old shoot internodes were collected and immediately frozen in dry ice. To improve RNA yield and quality, brown scale leaves were removed from flower buds prior to freezing. Phenological stages after flower bud break occurring at the time of collection were recorded as: swollen bud

(inner leaf scales are visible), green tip (sepals are visible), pink tip (petals are visible), full bloom (partially and fully opened flowers) and petal fall (adapted from University of California IPM, 1985). RNA was extracted according to Brunner et al. (2004) with minor modifications, treated with DNase I (Ambion® Turbo DNA-*free*™ Kit, Applied Biosystems, Carlsbad, CA, USA) and quantified using Nanodrop (Thermo Scientific). Temperature records were obtained from the nearest meteorological station (Lat. 38º 44' 35" N, Long. -9º 13' 13" W; www.wunderground.com).

2.2 Semi-quantitative RT-PCR

Two micrograms of total RNA were used for cDNA synthesis with Transcriptor High-Fidelity cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) in 10µl total volume. cDNA samples were diluted 1:3 in sterile miliQ water and used as template for PCR in 20µl total volume, using gene specific primers (Table 1) and 1U GoTaq® DNA Polymerase (Promega Corp., Madison, WI, USA), according to manufacturers' instructions. *PrdCBF* genes were amplified using 2µl cDNA template, and 1µl was used for the remaining genes under study. PCR was performed as follows: 5min incubation at 95ºC, followed by 24 to 35 cycles of 30sec at 95ºC, 30-40sec at corresponding annealing temperature (Table 1) and 40-60 sec at 72ºC, and a final extension step for 5min at 72ºC. Total reaction volumes were analyzed by electrophoresis on 1.2% agarose gel stained with ethidium bromide. Images were captured using the ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). Technical PCR replicates were repeated for each gene at least once. Amplicon intensities were quantified using ImageJ (http://imagej.nih.gov/ij/) and normalized for *PrdActin* or *PrdαTubulin*. Along the analyzed collection points, *PrdActin* expression showed to be more stable in shoots while *PrdαTubulin* showed less variation in flower buds. Relative expression of each gene was determined as the average of normalized band intensities detected for each time point,

in two to three replicate trees. This approach was used to better summarize the global expression of each gene in all trees analyzed.

Table 1. Primer sequences used for gene expression analysis by semi-quantitative RT-PCR and corresponding annealing temperatures (Anneal), number of PCR cycles and amplicon sizes (Ampl). Almond α-*Tubulin* and *Actin* genes were used as housekeeping genes.

Genes (Accession n.)		Primer sequence	Anneal (ºC)	PCR cycles	Ampl (bp)
PrdCBF1 (JQ317157)	F	5'-CGCTAATGAACAGGTTCTTCTCTCA-3'	58	32	550
	R	5'-TTCACACTATCCTTCTTCTTCTTCTTC-3'			
PrdCBF2 (JQ317158)	F	5'-CTCTAATGGACTTGTCTCAACTTTC-3'	58	32	540
	R	5'-CCAAGTTCACACTACCCTTCTTG-3'			
PrdDHN1 (JQ317156)	F	5'-TCGTACTTTGAAAAATGGCG-3'	62	23	329
	R	5'-TAGTAAACCCTTCTTCTCCTGGTG-3'			
PrdGA20OX (JQ412172)	F	5'-GAGCACTCTTTCTATTGGGATCAT-3'	58	28	559
	R	5'-TCAGCTGATTTTCTGTTGAAGCCA-3'			
PrdGA2OX (ppa008414m)*	F	5'-AAGCAGGGCAACCTAATCCT-3'	58	28	466
	R	5'-TGATCAGGTGGGACTGGAAT-3'			
PdMADS1 (AY947462)	F	5'-CGGAGTGGAAGCAAAAGTAAAGGT-3'	58	28	737
	R	5'-TAGCATTTGCTGAATCTCTCTCC-3'			
PrdMADS3 (AY947464)	F	5'-GATAATATTTTAGCTGGCAAGGA-3'	58	28	800
	R	5'-TTGGCAAGCTTTTATGACTGA-3'			
PrdActin1 (AM491134)	F	5'-AGCAAGGTCCAGACGAAGAA-3'	58	24	385
	R	5'-TGTAGGTGATGAAGCCCAATC-3'			
Prdα*Tubulin* (X67162)	F	5'-ATTGAGCGACCCACCTACAC-3'	58	24	428
	R	5'-GTGGGTGGCTGGTAGTTGAT-3'			

* International Peach Genome Initiative (www.rosaceae.org/peach/genome)

2.3 Flower bud development during ecodormancy

During year-II, the morphological development of flower buds was assessed during dormant and swollen bud stages, occurring from early November to late December. For each tree analyzed, four flower buds were randomly selected from different branches and longitudinal sections were observed using a Nikon SMZ800 stereomicroscope (2x amplification). Images were recorded using a Digital Sight Camera System (Nikon, Japan) coupled to the stereomicroscope.

3. Results

3.1 Seasonal development of almond trees in two consecutive years

To assess the putative role of *PrdCBF*s and other candidate genes during natural dormancy and cold acclimation period, three almond trees growing naturally in Monsanto forest were selected to study flower development along two consecutive years. These trees, although growing in a feral state, showed similar phenological development and early flowering habit. According to the temperature records, the collection period in year-II (2010/11) was on average colder than in year-I (2009/10), particularly during fall. In fact, chilling accumulation (number of hours below 7.2ºC [Weinberger, 1950]) started earlier in year-II, and was closely followed by that of year-I, throughout the sample collection period (Supplementary Figure 1). Timing of bud break in vegetative and flower buds was determined by macroscopic observation of bud morphology. In year-I, leaves started to emerge from vegetative buds mainly in late January while in year-II this occurred approximately two weeks earlier (Figure 1). In both years, flower bud break (occurrence of swollen buds, up to the emergence of inner green scales) was detected already during December (Table 2; Figure 1). During late December to early January, the phenological stages observed were closely similar in both years (Table 2). However, blooming (considered here as >50% anthesis) occurred earlier in year-II as compared to year-I (Table 2). The early fulfilment of chilling requirements and the mild temperature periods occurring during late December to early January in year-II (Supplementary Figure 2b) are suggested as promoters of this advanced development.

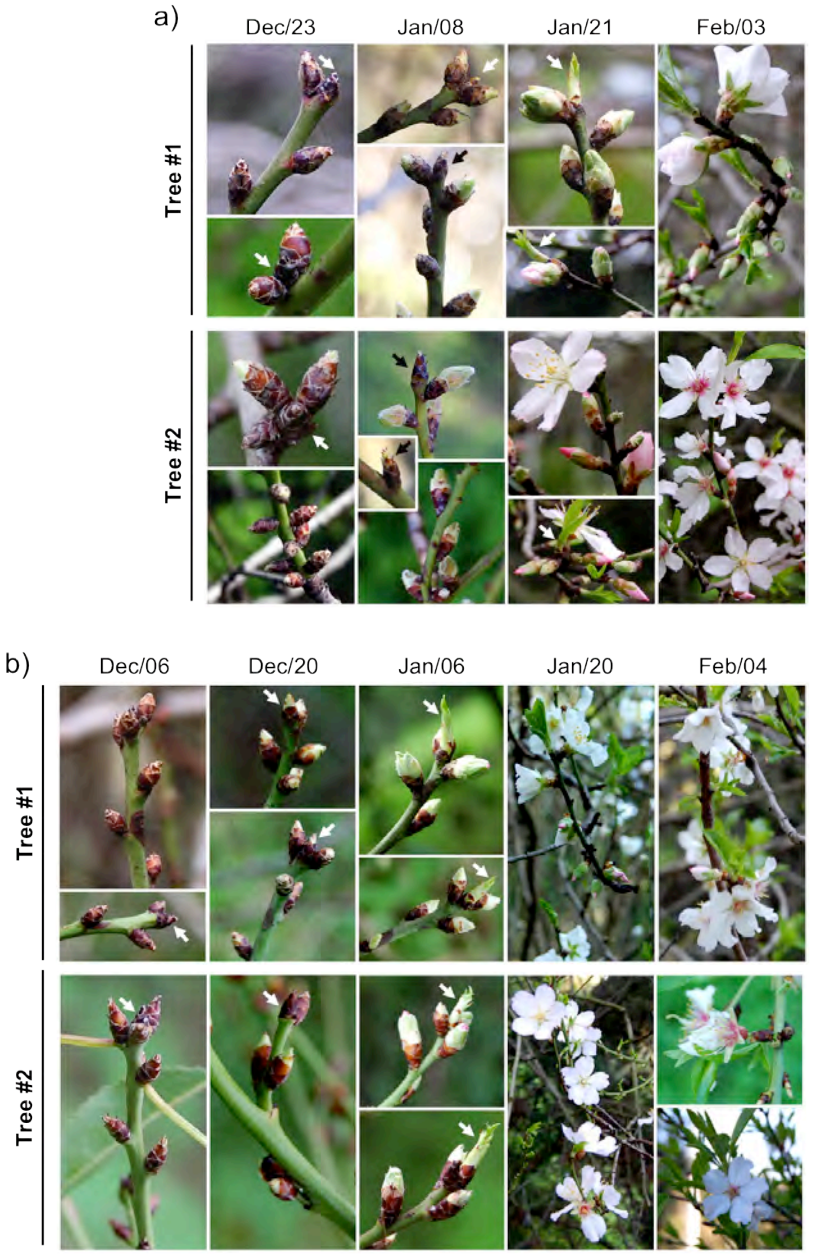

Figure 1. Developmental stages of vegetative and reproductive buds from two representative almond trees (#1 and #2) sampled in this study, growing in Monsanto (Lisboa, Portugal), in year-I (a) and year-II (b). Arrows indicate vegetative buds.

Table 2. Schematic representation of flower bud developmental stages, occurring during ecodormancy in both years in all trees sampled. Phenological development was divided as follows: a) swollen bud; b) green tip; c) pink tip; d) blooming; e) petal fall. Black squares represent the predominant stage (>50%) and grey represent other stages scored (<50%).

Stage	Dec 23	Jan 08	Jan 21	Feb 03	Dec 20	Jan 06	Jan 20	Feb 04
a)								
b)								
c)								
d)								
e)								
	Year-I				**Year-II**			

3.2 Expression pattern of PrdCBF*s in young shoots during natural cold acclimation*

Under controlled environmental conditions, *PrdCBF1* and *PrdCBF2* genes were shown to be induced upon low temperature exposure (below 12ºC) (Chapter II). To assess the putative role of *PrdCBF*s during natural cold acclimation, their seasonal transcript expression was studied in almond adult trees. One year-old shoot internodes were collected from late summer to mid-winter in regular intervals. The average gene expression was compared to the daily minimum temperature, which occurred prior to sample collection. During year-I, *PrdCBF1*, *PrdCBF2* and *PrdDHN* transcripts showed increased levels during mid-fall stages (after Nov/05), in agreement with the decrease in minimum temperatures to levels below 15ºC (Figure 2). After Jan/06, and up to the end of sample collection, the expression levels of *PrdCBF1*, *-2* and *PrdDHN1* appeared to decrease, although temperatures were still around 10ºC (Figure 2). However, these observations were hardly validated by statistical analysis due to the variability in expression occurring between trees. Nevertheless, replicate expression analysis conducted during year-II using two of the trees used in year-I showed again a putative

induction of, at least, *PrdCBF2* during early fall (Figure 3). Moreover, the transcript accumulation of *PrdCBF1*, *-2* and *PrdDHN1* also showed to decrease in late collection stages during January. In addition, *PrdCBF2* transcript accumulation showed an interesting variation, which agreed with the temperature variation recorded for specific dates, particularly from November to December (Figure 3). In both years, the decrease in gene expression during winter stages agreed with the timing of vegetative bud break.

3.3 PrdCBF*s expression is repressed after flower bud break*

Gene expression in flower buds was determined from November to February. In year-I, in the stages prior to bud break, expression of *PrdCBF1* and *-2* and *PrdDHN1* was somewhat constant (from November to early December, Figure 4). In year-II, expression of *PrdCBF1* and *-2* showed a higher variation between the three time points analyzed during the same period (Figure 5), which closely reflected differences in minimum temperatures recorded between collection dates. In fact the expression pattern of *PrdCBF2* in flower buds was very similar to that observed in shoot internodes during the same collection trial (Figure 3). These results seem to reinforce the active environmental regulation of *PrdCBF*s, especially *PrdCBF2*. During initial stages of bud break, occurring from late December to early January, *PrdCBF2* and *PrdDHN1* transcript levels were similar to previous stages, but significantly decreased thereafter, when petals started to emerge from the calyx (Figure 4, Table 2). A similar decrease was detected two weeks earlier in year-II (Figure 5), in agreement with the advanced phenological development observed in this year (Table 2). Thus, downregulation of *PrdCBF2* and *PrdDHN1* seems to anticipate the onset of anthesis. In turn, expression of *PrdCBF1* was not consistent between years, although it was also low prior and/or after full bloom stages (Figure 4; Figure 5). In both years, the last time point (early February) was characterized by a

sharp decrease in minimum temperatures. However, the transcript levels determined for *PrdCBF1*, *PrdCBF2* and *PrdDHN1* were lower than those observed during cold acclimation stages in fall, suggesting that cold signalling pathways mediated by both *PrdCBF*s (which may include regulation of *PrdDHN1*) are repressed after flower bud break. Interestingly, this repression agrees to some extent with the expression pattern of *PrdCBF*s and *PrdDHN1* determined for shoot internodes.

3.4 Expression of two MADS-box genes is induced after bud break

To correlate the expression pattern of both *PrdCBFs* with developmental changes occurring in flower buds during dormancy-activity cycles, the expression of two homeotic *MADS-box* genes was also studied. *PrdMADS1* is a D-type *MADS-box* gene, expressed in carpels (Silva et al., 2007), homologous to the peach *PPERSTK* (99% amino acid identity, data not shown) (Tani et al., 2009) and the *A. thaliana SEEDSTICK* (*STK*) (Rounsley et al., 1995), both specifically expressed during ovule development. *PrdMADS3* belongs to the E-type sub-group of MADS-box genes, which are specifically expressed in flowers in all the whorls (Silva et al., 2007). In both years, *PrdMADS1* transcript accumulation was only detected after bud break (Figures 6 and 7), mainly since the green-tip stage (Table 2). This late-stage expression pattern may correlate with the beginning of ovule development. In turn, *PrdMADS3* expression was detected at low levels during November to early December but was gradually upregulated during bud break up to the onset of blooming (Figures 6 and 7). These results revealed that both genes could be candidate markers of specific changes in tissue differentiation and specialization occurring after ecodormancy break up to anthesis.

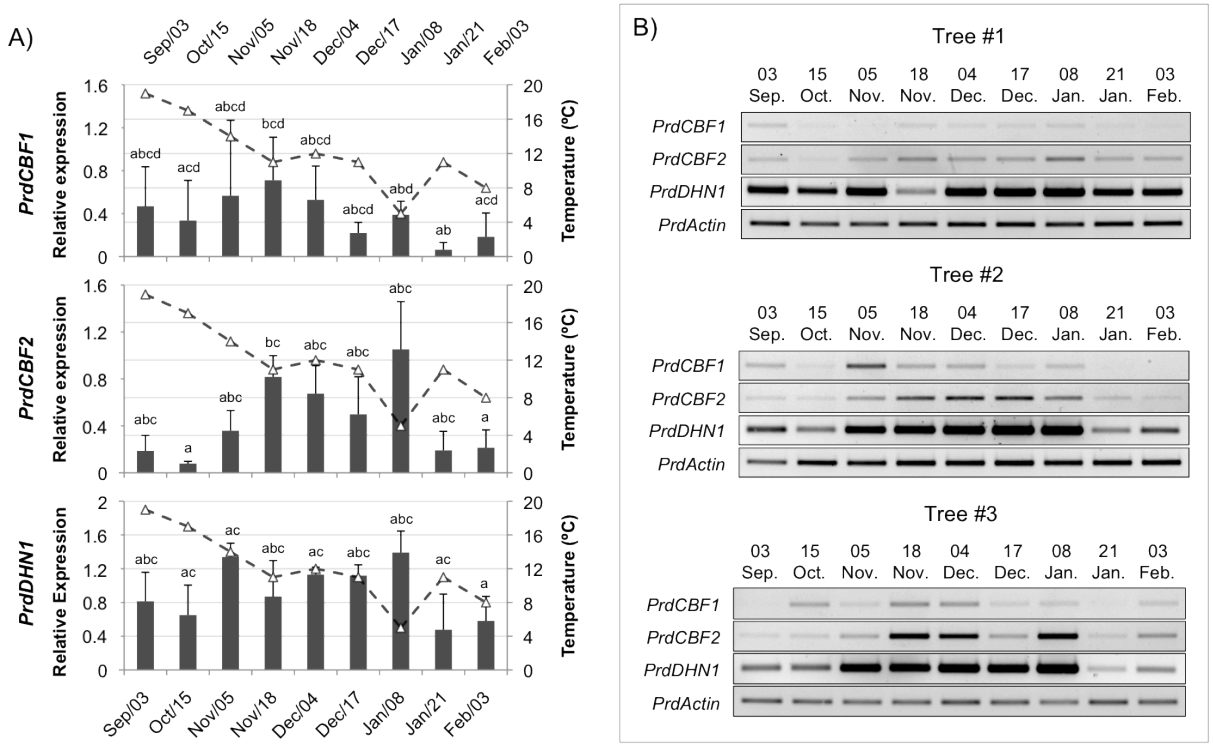

Figure 2. Seasonal gene expression patterns of *PrdCBF1*, *PrdCBF2* and *PrdDHN1* in one-year-old shoot internodes, collected from adult almond trees during late summer to early winter in year-I. (A) Transcription level for each gene was calculated by quantification of band intensities obtained from semi-quantitative RT-PCR and normalized for *PrdActin*. Data represents mean ± standard deviation (n=3). Different letters indicate statistical significant differences based on Student's *t* test ($p<0.05$). Open triangles represent the minimum temperatures determined for each date. (B) Representative gels used for band quantification are shown for each tree analyzed.

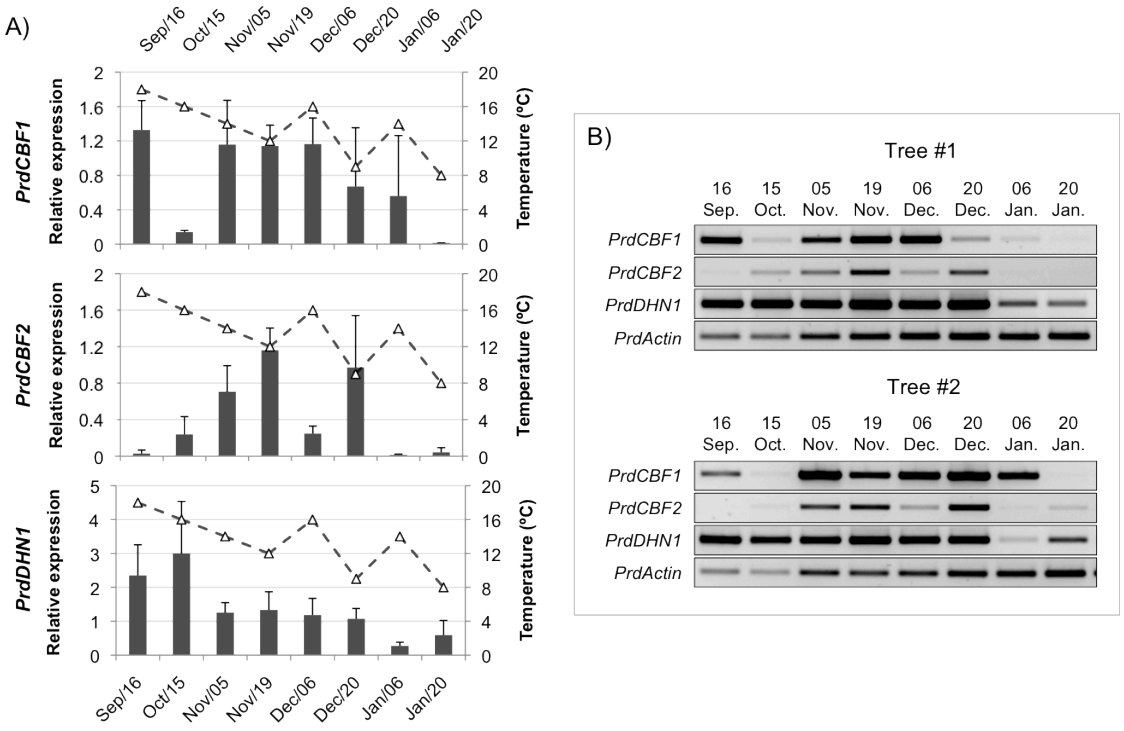

Figure 3. Seasonal gene expression patterns of *PrdCBF1*, *PrdCBF2* and *PrdDHN1* in one-year-old shoot internodes, collected from adult almond trees during late summer to early winter in year-II. (A) Transcription level for each gene was calculated by quantification of band intensities obtained from semi-quantitative RT-PCR and normalized for *PrdActin*. Data represents mean ± standard deviation (n=2). Open triangles represent the minimum temperatures determined for each date. (B) Representative gels used for band quantification are shown for each tree analyzed.

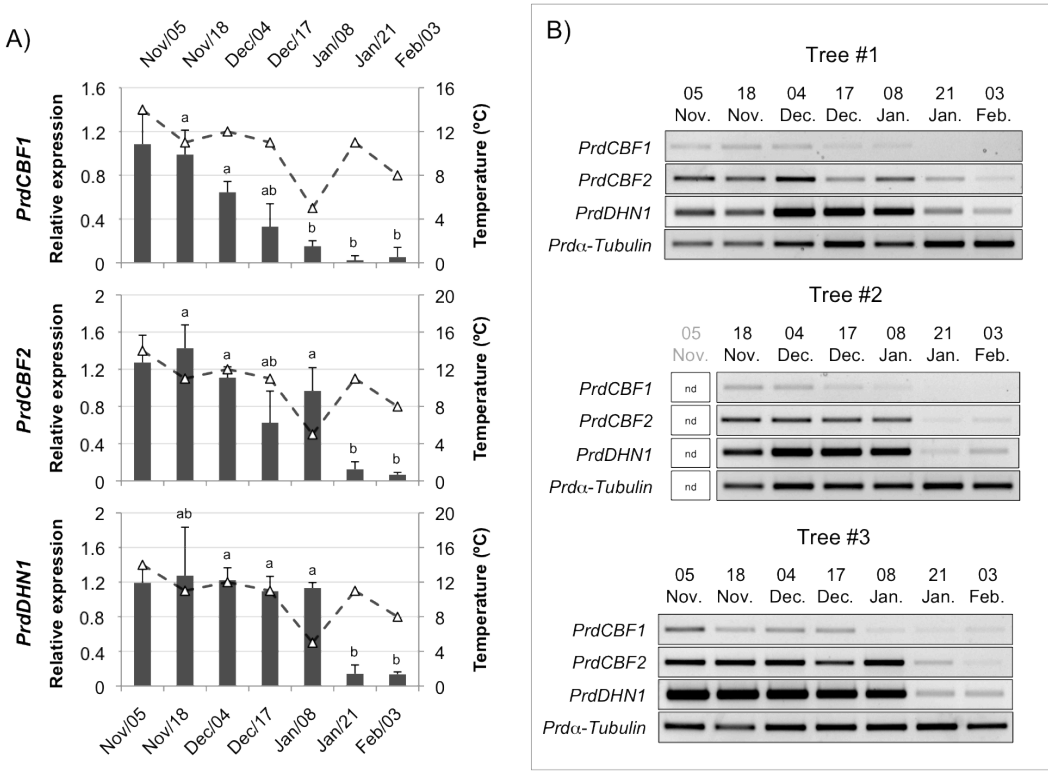

Figure 4. Seasonal gene expression patterns obtained for *PrdCBF1*, *PrdCBF*2 and *PrdDHN1* in flower bud samples, collected from adult almond trees during fall and early winter development in year-I. (A) Transcription level for each gene was calculated by quantification of band intensities obtained from semi-quantitative RT-PCR and normalized for *Prdα-Tubulin*. Data represents mean ± standard deviation (n=2 for Nov/05; n=3 for remaining collections dates). Different letters indicate statistical significant differences based on Student's *t* test ($p<0.05$) between time points with n=3 replicates. Minimum temperatures determined for each date are indicated as open triangles. (B) Representative gels used for band quantification are shown for each tree analyzed. nd – not determined.

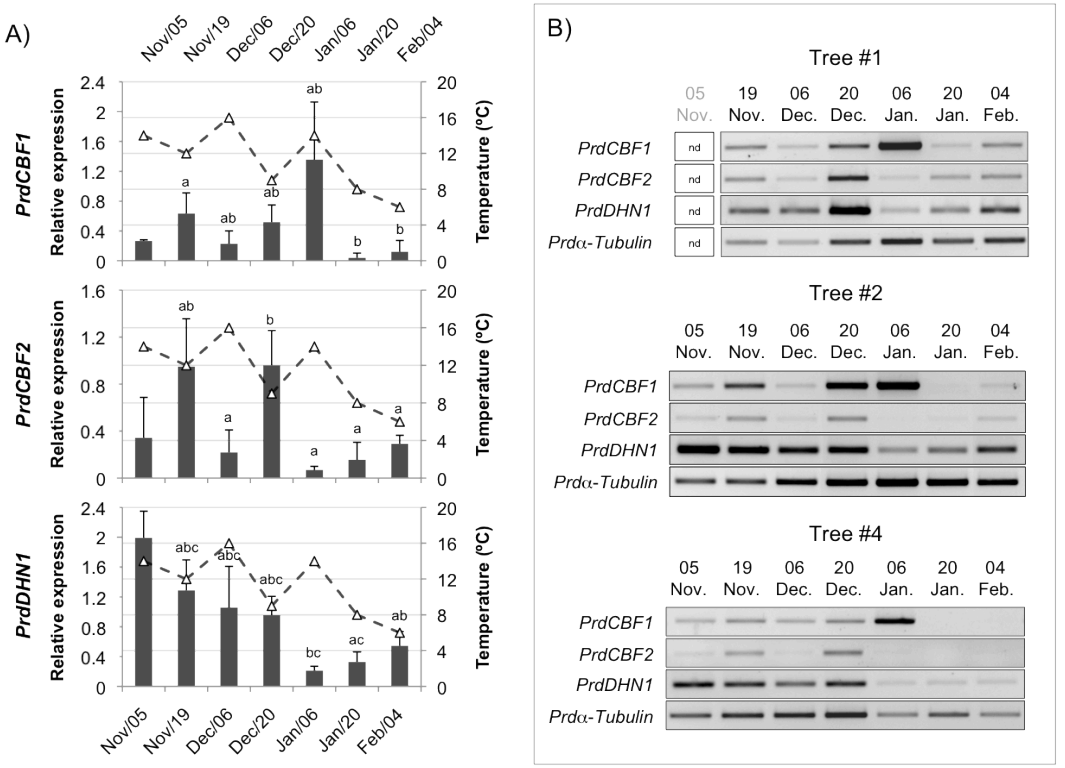

Figure 5. Seasonal gene expression patterns obtained for *PrdCBF1*, *PrdCBF2* and *PrdDHN1* in flower bud samples, collected from adult almond trees during fall and early winter development in year-II. (A) Transcription level for each gene was calculated by quantification of band intensities obtained from semi-quantitative RT-PCR and normalized for *Prdα-Tubulin*. Data represents mean ± standard deviation (n=2 for Nov/05; n=3 for remaining collections dates). Different letters indicate statistical significant differences based on Student's *t* test ($p<0.05$). Minimum temperatures determined for each date are indicated as open triangles. (B) Representative gels used for band quantification are shown for each tree analyzed. nd – not determined.

3.5 Two genes involved in GA metabolism are differentially regulated during bud break

GA metabolism plays an important role during bud dormancy induction and release. To elucidate the role of GAs in almond flower bud development, the expression of two genes for GA metabolism, *PrdGA20OX* and *PrdGA2OX* was followed from fall to winter, up to anthesis. *PrdGA20OX* is a close homologue of the GA 20-oxidase family in *Arabidopsis*, involved in the late steps of GA biosynthesis (Olszewski et al., 2002). In both years, *PrdGA20OX* expression was found prior to, and shortly after, bud break (Figures 6 and 7). However, in later stages, mainly after transition from green-tip to pink-tip stages (Table 2), *PrdGA20OX* transcript accumulation was reduced or not detected. Since this transition occurred later in year-I, *PrdGA20OX* transcription was still detected in early January (Figure 6), while in year-II expression levels were already reduced at this stage (Figure 7). Bioactive GAs are regulated by both the rate of their synthesis and the conversion into inactive forms, a reaction catalyzed by GA 2-oxidases (Olszewski et al., 2002). Expression of a *PrdGA2OX* candidate gene was observed in all the flower bud stages sampled in this study (Figures 6 and 7). Transcript levels seemed to increase along the assessed period, however this observation was not validated by statistical analysis, probably due to the variability in expression observed between trees. Nevertheless, the distinct expression pattern of both *PrdGA20OX* and *PrdGA2OX* suggests that during growth resumption, and prior to anthesis there is a genetic reprogramming of the GA metabolic pathway.

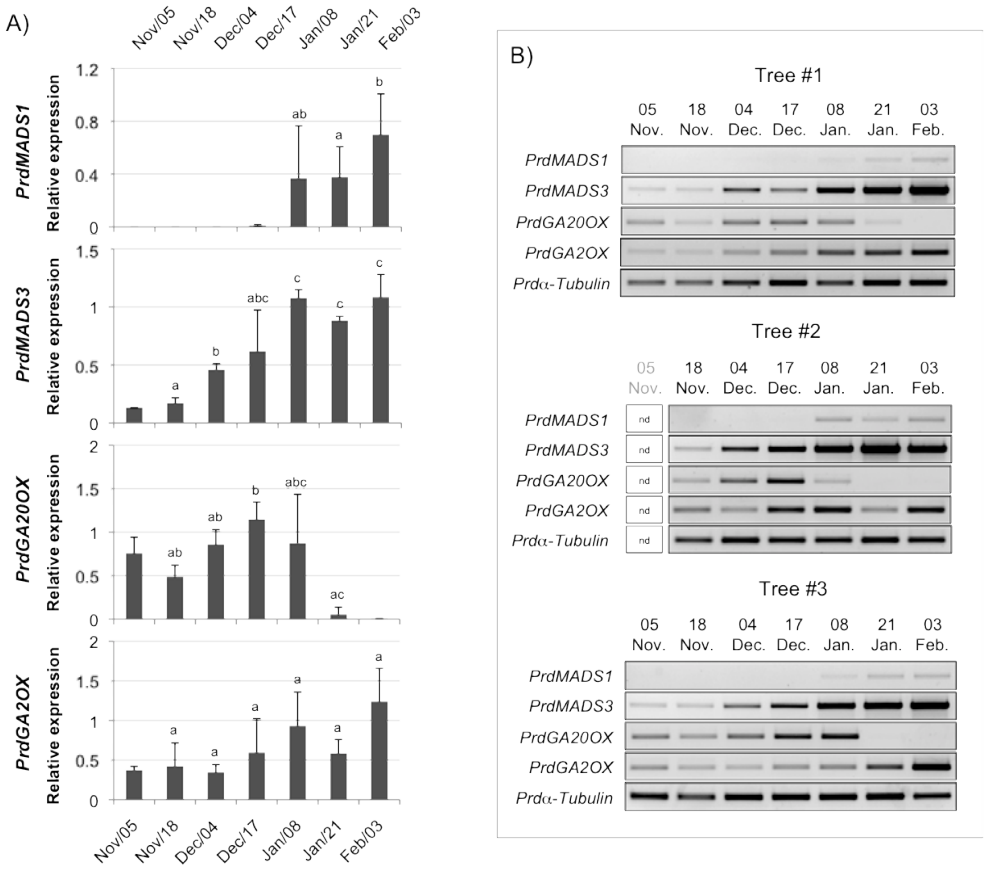

Figure 6. Expression analysis of genes related to floral organ development and GA metabolism in flower buds, collected from adult almond trees during fall and early winter development in year-I. (A) *PrdMADS1*, *PrdMADS3, PrdGA20OX* and *PrdGA2OX* transcript levels were determined by quantification of band intensities obtained from semi-quantitative RT-PCR, and normalized for *Prdα-Tubulin*. Data represents mean ± standard deviation (n=2 for Nov/05; n=3 for remaining time points). Different letters indicate statistical significant differences based on Student's *t* test ($p<0.05$) between time points with n=3 replicates. (B) Representative gels used for band quantification are shown for each tree analyzed. nd – not determined.

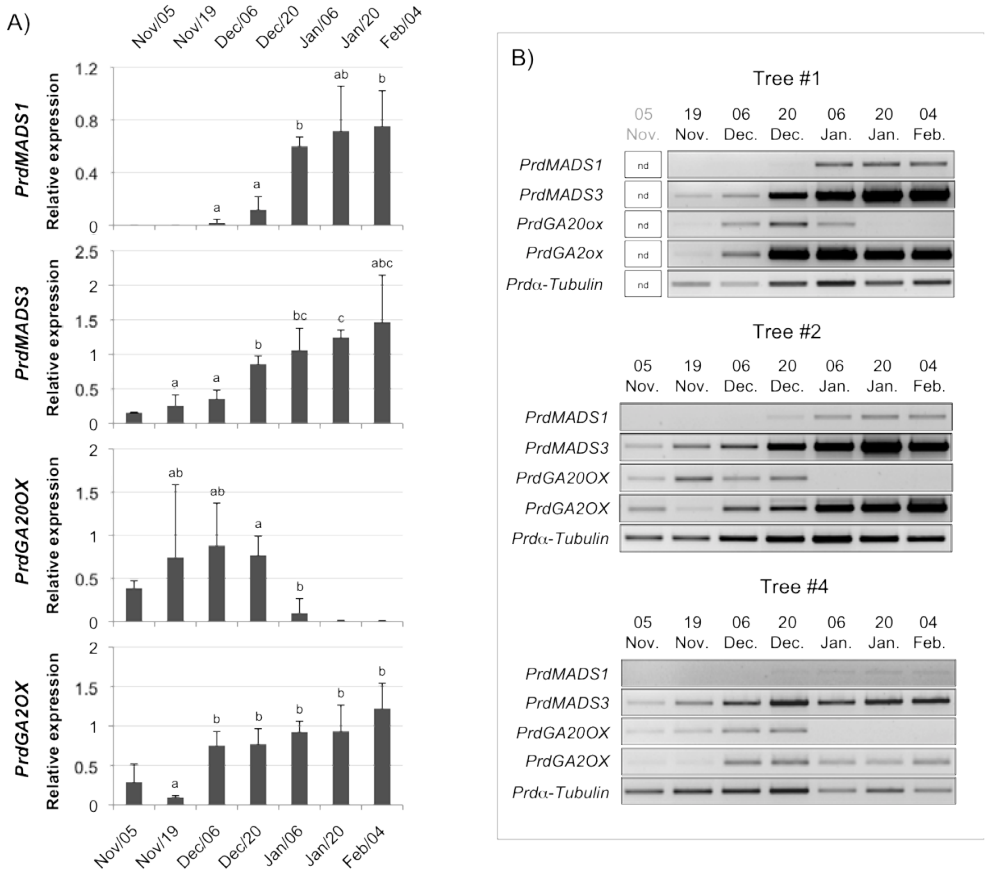

Figure 7. Expression analysis of genes related to floral organ development and GA metabolism in flower buds, collected from adult almond trees during fall and early winter development in year-II. (A) *PrdMADS1*, *PrdMADS3, PrdGA20OX* and *PrdGA2OX* transcript levels were determined by quantification of band intensities obtained from semi-quantitative RT-PCR, and normalized for *Prdα-Tubulin*. Data represents mean ± standard deviation (n=2 for Nov/05; n=3 for remaining time points). Different letters indicate statistical significant differences based on Student's *t* test ($p<0.05$) (B) Representative gels used for band quantification are shown for each tree analyzed. nd – not determined.

During year-II, flower organ development was examined in dormant buds up to bud break (Figure 8) in trees #1 and #2. This analysis revealed small but consistent differences between both trees. More particularly, at Dec/20 (and even in Dec/14 time point, which was not included the gene expression analysis), the change in anther colour, which relates to final stages of pollen grain development, occurred first in tree #2 (Figure 8). Notably, expression of *PrdGA20OX* in this tree was only detected up to this stage (Figure 7B). In tree #1, the deduced delay in polen development may also agree with the expression of *PrdGA20OX*, since it was detected up to Jan/06 (Figure 7B). Thus, *PrdGA20OX* could be further studied as a candidate marker for anther development.

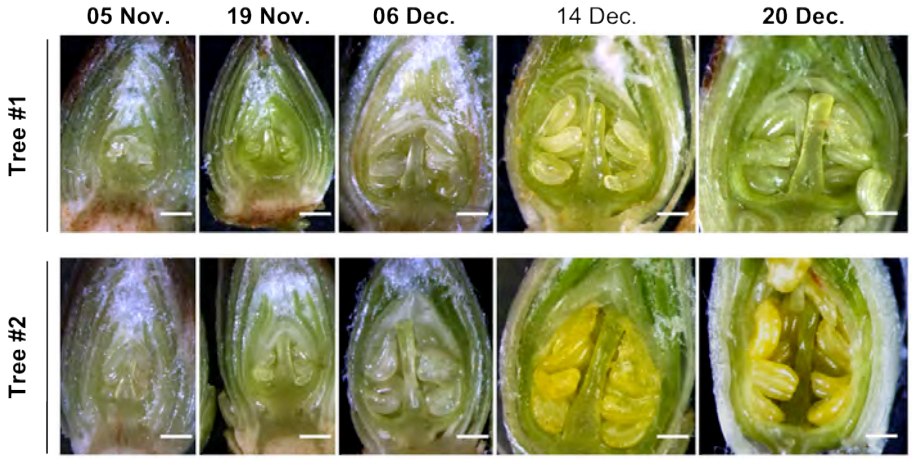

Figure 8. Representative morphological stages of floral development occurring from November to December in trees #1 and #2, during year-II. Scale bar represents 0.5mm.

4. Discussion

The involvement of the CBF TFs during winter dormancy has received some attention in perennial plants, particularly in temperate fruit trees. However, there is still little information regarding the role of these TFs over extended periods of time or during dormancy-activity transitions in perennials. In this study, the expression of *PrdCBF1* and *PrdCBF2* was

analyzed in tissue samples collected from adult almond trees growing in natural conditions.

In both years analyzed, the expression pattern observed for shoot internodes suggested a higher accumulation of *PrdCBF1* and *-2* during fall and early winter, in agreement with a decrease in average temperatures and the onset of chilling conditions. Expression of *PrdCBF1* was detected in late summer stages, while *PrdCBF2* expression was detected mainly after the onset of fall. Expression of the *PrdCBFs'* deduced target *PrdDHN1* was also detected at high levels during late summer. In poplar, SD-induced growth cessation and dormancy may be enough to activate the expression of several cold-regulated genes and increase cold hardiness, without prior exposure to cold temperatures (Welling et al., 2002; Druart et al., 2007; Rohde et al., 2007; Ruttink et al., 2007). In several *Prunus* species, the induction of growth cessation and dormancy by SD photoperiod is highly dependent on species/cultivar-specific low temperature perception (Heide, 2008). In peach, continuous growth was observed at a 23/17ºC day/night temperature in 16h photoperiod, while at the same temperatures, but with a photoperiod ≤ 13h, plants ceased growth and induced dormancy (Went 1957 in Heide 2008). In the current study, during the first half of September, the day-length was approximately 12h30, with average temperatures ranging from 29ºC (maximum) to 18ºC (minimum) (data not shown). These conditions may have been enough to trigger dormancy development in this species, but further confirmation is required. In peach, expression of the *PrdDHN1* homolog was also observed during late summer in bark and root tissues from field-grown trees (Artlip et al., 1997; Wisniewski et al., 2006 Bassett et al., 2009), but this gene was not responsive during dormancy induction in controlled conditions (SD treatment at 25ºC) (Wisniewski et al., 2006). These authors suggested that the lack of transcriptional activation of *PpDHN1* under these conditions could be related to insufficient decrease of water content under the tested conditions (Wisniewski et al., 2006). In turn,

the involvement of a peach CBF gene (*PpCBF1*) in SD-dependent dormancy induction was recently proposed (Wisniewski et al., 2011), but BLAST analysis on the peach genome (www.rosaceae.org/peach/genome) showed that *PrdCBF1* and *PpCBF1* are not direct homologs (Chapter II). Therefore, while the effect on dormancy induction may not yet be discarded, the expression of *PrdCBF1* and *PrdDHN1* during late summer could be related to other stress conditions, such as drought or oxidative stress.

During fall (October to December), *PrdCBF2* transcript accumulation in both shoot internodes and flower buds showed an association to the minimum temperatures in each time point assessed, with higher levels being detected when minimum temperatures were close to or below 12ºC. This observation was particularly obvious in year-II, in which minimum temperatures were more variable during that period. Under controlled conditions *CBF* genes are rapidly upregulated by cold, returning to lower/basal levels within 24h of exposure to constant temperature (Benedict et al., 2006; El Kayal et al., 2006; Welling and Palva, 2008; Navarro et al., 2009; Wisniewski et al., 2011; Chapter II). In the present study, samples were collected 3-4h after dawn (shortly after the night minimal temperatures). However, under field conditions, it is difficult to conclude if the measured transcript levels of *PrdCBF2* result from the minimum temperature reached prior sample collection or from the temperature decline verified along the previous night. Nevertheless, *PrdCBF2* appears to be an important player in temperature signalling in almond, during the cold acclimation initiated in fall.

Bud break and growth resumption in vegetative and reproductive buds occurred very early in the almond trees under study, confirming their reduced temperature requirements for dormancy break. Flower bud break was characterized by the transcriptional induction of two floral homeotic genes, *PrdMADS1* and *PrdMADS3*. These genes are candidate members of the flower MADS-box gene family, which includes well-known players

regulating meristem identity and floral organ development in model plants, organized according to the ABCDE model (Immink et al., 2010). *PrdMADS1* is a carpel-specific expressed gene (Silva et al., 2007), homolog of PPERSTK, which belongs to the ovule specific D-class of MADS-box genes in peach (Tani et al., 2009). In *Arabidopsis*, the homolog *STK* gene is required for normal development of the funiculus, during the emergence of ovule primordia (Pinyopich et al., 2003). In turn, *PrdMADS3* belongs to the E-class, also known as *SEPALLATA (SEP)* genes, which are necessary for the function of A-, B- and C-class genes (Pelaz et al., 2000; Honma and Goto, 2001; Ditta et al., 2004), being expressed in all floral whorls (Silva et al., 2007). According to Reinoso et al. (2002*a*), peach flower buds show continuous development during late autumn and winter. Sterile whorls differentiate rapidly in late summer through early fall, arresting their development in fall/winter dormancy, while the reproductive organs differentiate and develop throughout winter (Reinoso et al., 2002*a*). Assuming a similar developmental pattern in almond, those morphological changes could relate to the residual, but increasing, expression level of *PrdMADS3* observed during November and early December. The high transcript levels observed after bud break may support the rapid development of sepals and petals, leading to anthesis. In *Prunus* spp., megasporogenesis and further ovule maturation is often observed close to, or during, anthesis (Alburquerque et al., 2002, Ruiz et al., 2010). Thus, different stages of ovule maturity at anthesis are often observed even between cultivars (Pimienta and Polito 1983; Egea and Burgos 2000; Alburquerque et al., 2002; Egea and Burgos 2000, Ruiz et al., 2010), which may impact fertilization and fruit set. In the present study, *PrdMADS1* transcription was only detected in the final stages of development, when flower buds were in the green-tip stage. This suggests that *PrdMADS1* could be an interesting marker of ovule emergence.

Exogenous applications of GAs often induce dormancy break in a wide variety of woody angiosperms (Looney, 1997). However, GA application in flower buds may have contrasting effects on bud break (induction or inhibition), depending on the timing of treatment and probably related to the dynamics of GA metabolism along dormancy (Reinoso et al., 2002*b*). In peach, Luna et al. (1993) reported that levels of GA_1 and GA_3 reached maximum levels when the flower buds were at their lowest rate of organ development. According to these authors, prior to anthesis, lower levels of $GA_{1/3}$ were detected, although their likely precursor GA_{20}, and also GA inactive forms ($GA_{8/29}$), showed to increase. In model plants, the production of GA_{20} is related to the activity of GA 20-Oxidases, while GA 2-Oxidases mediate the production of $GA_{8/29}$ (Achard and Genschik, 2009). In almond, the expression of *PrdGA20OX* in flower buds was observed prior and after bud break, being downregulated prior to anthesis. This expression pattern may agree with the expression of several *GA 20-Oxidase* genes in poplar, which were shown to increase in response to chilling exposure and during early phase of growth reactivation (Karlberg et al., 2010; Rinne et al., 2011). Conversely, transcription of *PrdGA2OX* was detected during all the assessed period, even when *PrdGA20OX* transcript levels were clearly reduced. Although these results cannot be directly correlated to endogenous GA levels, these patterns of expression fit, to some extent, in the dynamics of endogenous GAs previously determined for peach (Luna et al., 1993). It is thus suggested that after flower bud break there is a change in GA metabolism resulting from the differential regulation of, at least, *PrdGA20OX* and *PrdGA2OX*. In *Arabidopsis,* GAs are involved in the regulation of cell elongation in stamen filament as well as in cellular development in anthers (Cheng et al., 2004). In addition, stamens are major sites of GA production in flowers (Hirano et al., 2008; Hu et al., 2008), and this fact has been implicated in the control of corolla expansion and pigmentation during anthesis (Weiss and Halevy, 1989; Hu et al., 2008). In

the present study, analysis of flower bud morphology allowed a preliminary association of anther maturation with the expression of *PrdGA20OX*. In fact, when transcript levels of this gene showed to decline (early winter), anthers were reaching full maturity. Thus, it would be interesting to further confirm this association and investigate a possible correlation with the timing of anthesis.

In winter, when a visible increase in bud activity was observed, the expression of both *PrdCBFs* and *PrdDHN1* reached lower levels, as compared to those observed in previous stages. More particularly, the coordinated downregulation of *PrdCBF2* and *PrdDHN1* transcription was observed in internode and flower bud samples, mainly after bud break in both vegetative and reproductive buds. When comparing both years, a two-weeks advance (at least) in leaf and flower emergence was also observed in year-II, which correlated with the timing of *PrdCBF2* and *PrdDHN1* downregulation in January. However, minimum temperatures recorded in winter collection stages were in some cases lower than in fall. Thus, these results strongly suggest that cold signalling pathways mediated by *PrdCBFs* (which may include *PrdDHN1*) are damped in winter in both internode and flower bud tissues during growth resumption. Recent evidences demonstrated the involvement of *DAM* (*DORMANCY ASSOCIATED MADS-box*) genes in dormancy regulation in peach (Li et al., 2009, Yamane et al., 2011). More particularly, *PpDAM5* and *PpDAM6* genes were proposed to function as a dose-dependent growth inhibitor signal in ecodormant vegetative buds (Yamane et al., 2011). The presence of CBF-specific CTR/DRE *cis*-elements in promoters of *PpDAM5* and *-6* genes suggests their association to the CBF regulon (Yamane et al., 2011, Wisniewski et al., 2011). To some extent, this agrees with their transcriptional induction observed in vegetative buds under low temperature treatments during early dormancy stages (Yamane et al., 2011). However, these genes also showed to be downregulated in later dormant stages, in response to

extended chilling under controlled conditions (Jiménez et al., 2010, Yamane et al., 2011). Thus, it would be interesting to further investigate if downregulation of both *PrdCBFs* in winter is also caused by prolonged chilling exposure.

Expression of *PrdDHN1* in fall/winter development closely resembles that observed for *PpDHN1* in peach bark tissues (Artlip et al., 1997, Wisniewski et al., 2006). In this species, distinct patterns of expression were observed between sibling evergrowing (non-dormant) and deciduous genotypes, since in the former, the induction of *PpDHN1* was delayed in fall and its accumulation declined earlier in winter (Artlip et al., 1997). This pattern was associated with the absence of terminal growth cessation and to the reduced levels of cold hardiness observed in the evergrowing plants (Arora et al., 1992, Artlip et al., 1997). The almond trees analyzed in the present study showed terminal and axillary bud set and growth cessation, but *PrdDHN1* expression also showed to decline in early winter. Although the levels of cold hardiness of the studied trees during seasonal dormancy were not assessed, according to temperature records, the decline in *PrdCBF1, PrdCBF2* and *PrdDHN1* expression after bud break followed, in both years, a period in which temperatures reached deep chilling and increased thereafter (Supplementary Figure 2). As reviewed by Kalberger et al., (2006) resistance to deacclimation tends to decrease during dormancy-activity transitions, and loss of cold hardiness is usually associated with growth resumption. The negative effects of growth and development in cold hardiness are related to changes in sub-cellular structure caused, for example by the increase in water content and increased vacuolar volume that accompanies cell expansion (Kalberger et al., 2006). These changes will greatly increase frost sensitivity and plant's ability to re-induce cold acclimation is further compromised, since active growth may also compete for energy resources. Thus, the negative regulation of stress-responsive

genes (including CBF regulons), which are usually involved in defining chilling response, is also likely to occur during growth initiation.

The present study provides the first molecular evidence for the role of CBF-mediated low-temperature signalling in fruit tree flower buds, also pointing for putative markers to study the complex network of events occurring prior to anthesis. The results are summarized in a comprehensive scheme in Figure 9. During cold acclimation stage in fall, which will overlap sequentially with endo- and ecodormancy periods, *PrdCBF2* may play an active role in temperature signalling, responding to the temperature variations occurring during these stages, and likely contributing to maintain the active expression of *PrdDHN1* (and other members of the regulon). In turn, the role of *PrdCBF1* is still inconclusive since it may be regulated by additional factors. During ecodormancy, while chilling exposure represses growth, the occurrence of promotive temperatures induce growth and lead to a decrease in cold hardiness, which may be greatly due to a decrease in the expression of cold-regulated genes (or at least the *PrdCBF*s' regulon). The timing of bud break and growth resumption is also pinpointed by the specific upregulation of *PrdMADS1* and *PrdMADS3*, related to floral organ development, and by the downregulation of *PrdGA20OX*, which could be a prerequisite for anthesis. At this stage, reacclimation capacity also seems to be hampered due to active growth (Kalberger et al., 2006), while re-induction of *PrdCBF1* and *-2* in response to chilling may be repressed.

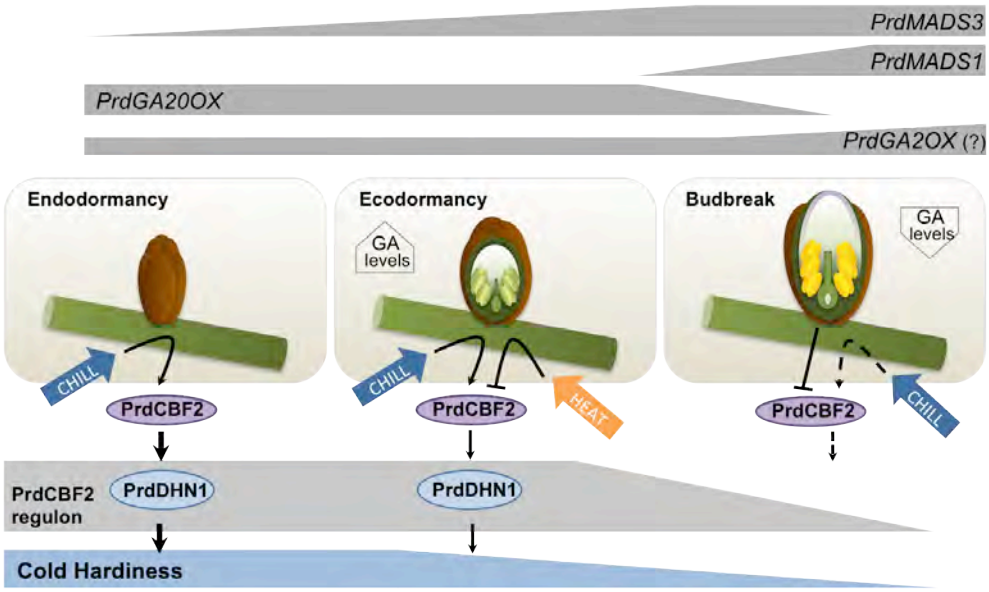

Figure 9. Schematic representation of the control of cold acclimation and dormancy break in almond flower buds and shoots. *PrdCBF2* may be involved in cold acclimation after endodormancy induction, playing a role in environmental signalling, mainly through low temperatures. Chilling temperatures (CHILL) play a role in determining the timing of endodormancy break and reestablishment of growth ability, according to plant specific chilling requirements. During the following ecodormancy stage, chilling represses growth until warm promoting temperatures (HEAT) occur. When temperature requirements for ecodormancy break are met, growth resumption occurs leading to bud break. At this stage, plants ability to maintain cold hardiness decreases (Kalberger *et al.*, 2006) which is likely induced by the downregulation of *PrdCBF2* and *PrdDHN1* expression, among other putative members of *PrdCBF2* regulon. After this stage, chilling temperatures may occur but this may only have a small effect on the induction of *PrdCBF2* and *PrdDHN1* (dashed arrows). In flower buds, bud break is characterized by an increase in *PrdMADS3* expression. In later stages, prior to anthesis, *PrdMADS1* is also induced, in agreement with the predicted timing of ovule formation. During ecodormancy and bud break stages there are also changes in GA levels (arrow boxes, Luna *et al.*, 1998), which was supported by the decreased expression of *PrdGA20OX,* a candidate gene in GA biosynthesis. The putative induction of *PrdGA2OX* expression (associated with GA catabolism) after bud break requires further confirmation.

5. Acknowledgements

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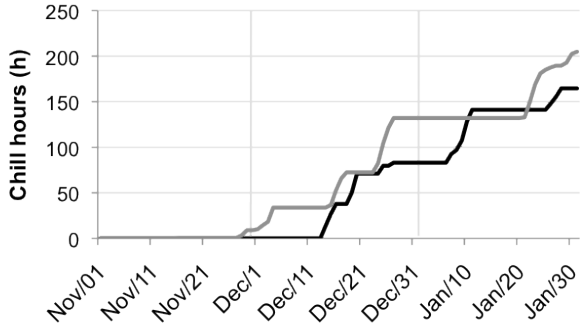
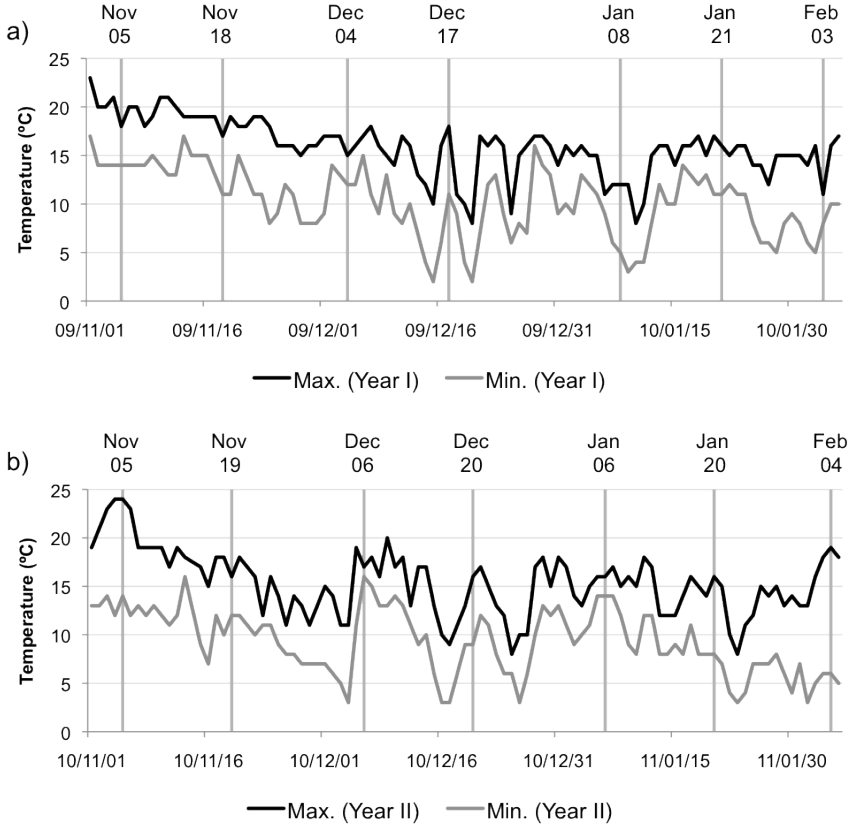
7. Supplementary Data



Supplementary Figure 1. Cumulative hours below 7.2ºC (chill hours) measured during the dormancy period in year-I (2009/2010, black line) and year-II (2010/2011, grey line).



Supplementary Figure 2. Maximum (black) and minimum (grey) temperatures (ºC) recorded from November to February, in (a) year-I and (b) year-II.

CHAPTER V

DNA Methylation as a Player in Organ-Specific Transcription of *PrdCBF2* in Flower Buds

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Summary

PrdCBF2 was proposed to be a player in low temperature perception and cold acclimation in almond. However, still during winter, adult trees showed a decrease in *PrdCBF2* transcript accumulation, after bud break, as compared to previous developmental stages. To investigate if low temperature response is differentially regulated along seasonal development at the epigenetic level we determined and compared the methylation pattern of *PrdCBF2* promoter from two different developmental stages in flower buds (dormant buds and open flowers). Using bisulfite sequencing, we detected low levels of cytosine methylation in the targeted promoter region and no consistent differences between both developmental stages. Nevertheless, a particular set of clones obtained from both DNA samples revealed the presence of a specific methylation target site. A preliminary gene expression analysis in specific floral tissues indicated that *PrdCBF2* transcripts are more abundant in sepals than in the remaining floral whorls. Thus, the occurrence of methylation within that promoter target site may be related to the regulation of *PrdCBF2* gene expression in specific floral organs.

1. Introduction

Throughout development, plants interact with the surrounding environment by reprogramming gene expression through a combination of molecular and biochemical mechanisms. Gene expression regulation relies on transcription factors and other regulatory proteins and also on epigenetic modifications, i.e. chemical modifications of histones and DNA, both associated with chromatin remodelling. Besides controlling the overall chromatin organization, these modifications play an important role in gating the access of effector molecules related to transcription, such as protein-complex machineries, to specific regulatory DNA sequences (Bannister and Kouzarides, 2011). Therefore, epigenetics, like any other gene regulatory mechanism, needs to be highly dynamic in response to environmental cues and developmental changes.

DNA methylation is a conserved epigenetic mark involved in genome defense against endogenous transposable elements or viral DNA and in regulation of gene expression (Meyer, 2010; Zhang et al., 2010). This epigenetic modification does not involve changes in the nucleotide sequence, but chemical modifications of a specific residue, cytosine. The addition of a methyl group to cytosine residues is mainly catalyzed by methyltransferases (MTases) and in plants this may occur in both symmetric (CG and CHG, where H can be C, T or A) and asymmetric (CHH) sequence contexts (Zhang et al., 2010). Four main families of plant MTases have been identified so far, having a role in *de novo* and/or maintenance methylation: DOMAINS-REARRANGED METHYL-TRANSFERASE (DRM) related to CHH methylation; METHYLTRANSFERASE 1 (MET1) responsible for most CG methylation and maintenance of non-CG methylation; CHROMOMETHYLASE (CMT), related to CHG methylation; and DNA methyltransferase homologue 2 (Dnmt2) whose role in methylation is still poorly understood (Zhang et al., 2010). Maintenance

methylation occurs after DNA replication, in the newly synthesized strand on hemimethylated DNA regions (methylation of the template DNA strand) (Chen and Li, 2004). When a methyl group is added to cytosine residues in nonmethylated DNA, this is referred to as *de novo* methylation (Chen and Li, 2004). Additionally, DNA methylation is modulated by other mechanisms, such as RNA-directed DNA methylation (RdDM), mediated by small interference RNAs (siRNAs) (Chan et al., 2005) and chromatin remodelling factors (e.g. histone methyltransferases [Johnson et al., 2007] and histone deacetylases [Zhang et al., 2010]). To some extent, these regulatory pathways provide a dynamic platform for the establishment of DNA methylation patterns, which may be crucial to guarantee the epigenomic plasticity needed for an efficient plant response to both developmental cues and environmental stress.

The mechanistic effect of DNA methylation in transcription is still unclear and several lines of evidence suggest that the roles of cytosine methylation are equally diverse and likely individualized for different genes (Zhang et al., 2010). Methylated cytosines may attract methyl-binding proteins, which in turn recruit histone modifiers and other chromatin remodelling proteins, forming a complex that can perturb the binding of TFs (Fransz and de Jong, 2002). High-resolution mapping of DNA methylation have uncovered some common aspects related to its distribution along plant genomes (Zhang et al., 2006; Zilberman et al., 2007; Li et al., 2008; Yan et al., 2010) also elucidating its role in chromatin organization. Transcriptionally inactive heterochromatic regions, which enclose highly abundant transposable elements (TEs) and repetitive sequences, contain the highest density of methylated cytosines. In euchromatic regions, lower but still significant levels of cytosine methylation were found. In this case, DNA methylation was abundant in transcribed regions, and was particularly low at the transcription start site (Zhang et al., 2006; Zilberman et al., 2007; Li et al., 2008; Yan et al., 2010). In fact, in *Arabidopsis*, the extent of

methylation within the gene body was correlated to transcript elongation rather than transcription initiation efficiencies (Zilberman et al., 2007).

Methylation within gene promoter regions, although less abundant in *Arabidopsis*, showed to be enriched in other plants like in poplar (*Populus trichocarpa*), increasing its frequency upstream the transcriptional start site (Feng et al., 2010). Additionally, site-specific methylation in non-coding regulatory regions, such as promoters, has been implicated in expression regulation of specific genes in different developmental stages or tissues (Baek et al., 2011; Soppe et al., 2000; Shibuya et al., 2009; Liu et al., 2004). For example, the methylation pattern of a putative siRNA target-region located in the promoter of a vacuolar Na^+/H^+ transporter gene is important for its differential expression in roots and leaves, and may affect salt sensitivity in *Arabidopsis* (Baek et al., 2011). Additionally, an endosperm-specific gene is silenced in other tissues by DNA methylation in the promoter, due to the action of siRNAs (Soppe et al., 2000).

Seasonal expression studies conducted in adult almond trees during natural environment (Chapter IV) revealed that transcript accumulation of cold-responsive *PrdCBF2* gene was high during fall (cold-acclimation stage), but decreased in winter in both shoots and mature flower tissues. Since during this late stage, minimum temperatures remained close to chilling levels (15ºC), we thought it would be relevant to check if the changes in *PrdCBF2* expression could be related to differences in the DNA methylation pattern within the corresponding promoter region. Thus, we have performed bisulfite sequencing of a specific genomic region upstream *PrdCBF2* using DNA samples obtained from flower buds in two different stages (dormant buds in fall and blooming in winter). DNA treatment with bisulfite converts unmethylated cytosines to uracils, keeping the methylated cytosines unchanged (Frommer et al., 1992). A target region of interest is then amplified by PCR, sequenced and then compared to the unconverted original template. In this study, we have not detected significant differences

on the methylation of *PrdCBF2* promoter in both developmental stages. Nevertheless we identified a conserved methylation pattern, which was suggested to be related to tissue/organ-specific regulation of transcription.

2. Materials and Methods

2.1 Bisulfite Sequencing

Genomic DNA from flower bud tissues collected at Dec/04 and Jan/21 (year-I, 2009/2010, tree #2), representing dormant bud and blooming stages, respectively, were obtained with the DNeasy® Plant Mini Kit (Qiagen). Bisulfite conversion was performed using the EpiTect® Bisulfite Kit (Qiagen), according to manufacturers' instructions, with 200ng of DNA. The bisulfite conversion thermal cycling conditions were as follows: 95ºC for 5 min, 60ºC for 25 min, 90ºC for 5 min, 60ºC for 85 min, 99ºC for 5 min, 60ºC for 175 min and 20ºC overnight. Two independent DNA conversions were performed for each DNA sample. Converted DNA (2µL) was used as template for PCR using primers targeting a 443bp region of *PrdCBF2* promoter, upstream TATA-box (MethCBF2-F, 5'-TTGATGAGTTAATATGATGGTAG-3'; MethCBF2-R, 5'-CTTARCAARCTCCCACTACAAAACA-3' [R=A/G]). PCR was performed in 20µL total volume with 1x GoTaq reaction buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.5µM of each primer and 1U GoTaq® DNA Polymerase (Promega). Reactions were incubated for 5 min at 95ºC followed by 30 cycles of 30 sec at 94ºC, 30 sec at 58ºC and 35 sec at 72ºC, with the final extension step for 5 min at 72ºC. Specific fragments from two independent PCR reactions were cloned into pCR2.1 and approximately 20 clones were randomly selected and sequenced (Beckman Coulter Genomics, Essex, UK). Sequences resulting from bisulfite-converted DNA were edited with EditSeq (DNASTAR Inc.) compared to the unconverted reference sequence and analyzed using Kismeth software (Gruntman et al., 2008).

2.2 Organ-specific expression of PrdCBF1, PrdCBF2 and PrdDHN1 genes

Calyx and inner floral whorl (corolla, stamens and pistils) samples were separated (post-freezing) from almond flowers collected in January, and were used for tissue-specific gene expression analysis. This was performed for flower bud samples from tree #2 in year-I (Jan/21) and repeated in tree #1 in year-II (Jan/20). RNA extractions and semi-quantitative RT-PCR for *PrdCBF1, PrdCBF2* and *PrdDHN1* were performed according to previously described in Materials and Methods section from Chapter IV. Expression of *PrdCBF1* and *PrdCBF2* genes was analyzed by semi-quantitative RT-PCR using 33 amplification cycles.

3. Results

3.1 Bisulfite sequencing of a PrdCBF2 *promoter region*

To determine if transcriptional repression of *PrdCBF2*, observed during ecodormancy break at late January (Figure 4 in Chapter IV) could be related to changes in DNA methylation, we analyzed the methylation pattern of a *PrdCBF2* promoter region in flower buds in two developmental stages: Dec/04, dormant bud; Jan/21, blooming stage. Given the restrictions of primer design for PCR amplification from bisulfite-treated DNA (due to conversion of unmethylated cytosines to uracils), this promoter region was selected among the overall suggestions given by Kismeth software, as the most informative (considering the previously predicted distribution of *cis*-elements, see Figure 4b in Chapter II). Therefore, bisulfite-converted DNA was used as template for PCR using primers targeting a -556 to -113bp region upstream the deduced TATA-box. For each DNA sample, around 40 clones obtained from two independent conversion reactions were sequenced, (41 for Dec/04 and 39 for Jan/21). After sequence analysis and comparison to the unconverted template sequence, it was observed that DNA methylation level in the target region was very low, with at least 6 out of 20 clones showing full conversion of unmethylated cytosines. In the

remaining clones, methylated cytosines occurred mainly in a stochastic manner, from one to seven cytosines per clone (data not shown). No significant difference in global or site-specific methylation was observed between DNA samples from the two developmental stages (Table 1 and Figure 1).

Table 1. Frequencies of methylated and unmethylated cytosines at CG, CHG and CHH sites within the analyzed *PrdCBF2* promoter sequence, determined for DNA samples collected in Dec/04 and Jan/21 (year-I).

	Site	Methylated	Unmethylated	Total
Dec/04	CG	1.62%	98.37%	491
	CHG	2.03%	97.96%	492
	CHH	1.85%	98.14%	2540
	All	1.85%	98.15%	3523
Jan/21	CG	1.28%	98.71%	468
	CHG	1.28%	98.71%	468
	CHH	1.98%	98.01%	2416
	All	1.79%	98.21%	3352

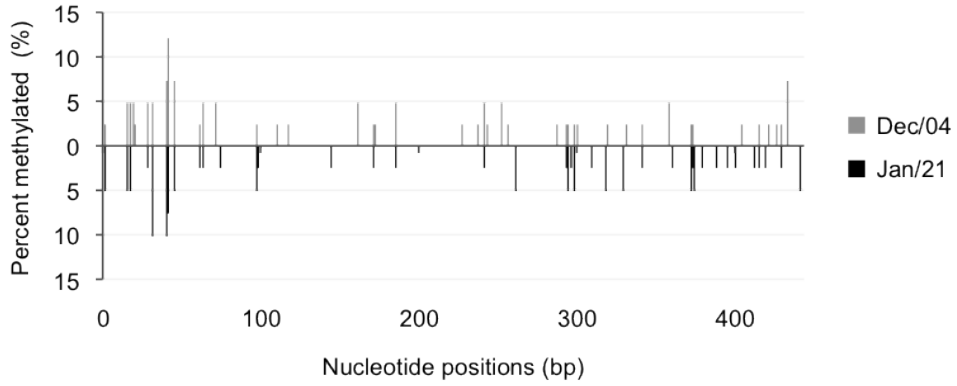

Figure 1. Methylation frequencies at each cytosine position within *PrdCBF2* promoter fragment targeted for bisulfite sequencing, obtained from flower bud DNA samples collected at Dec/04 and Jan/21.

These results suggest that transcriptional repression observed in late stages of flower bud development did not involve a significant

rearrangement in DNA methylation. However, it was observed that a specific region contained methylated cytosines in approximately 6-10% of the clones in both samplings (Figure 1). In fact, when analyzing all the sequences as a unique pool, a consistent methylation-enriched region was found in the 5' upstream region, particularly occurring in 3 CHH and 1 CHG sites (Supplementary Figure 1). This pattern was found to be exclusive of a particular set of clones (Supplementary Figure 2). Considering that DNA was extracted from the whole buds, we hypothesize that this methylation pattern could be related to tissue/organ-specific regulation of *PrdCBF2*, which could have lead to an underrepresentation of that putative core region. Putative binding sites related to MYB and WRKY TFs were predicted within this region, (Figure 4b [from -543bp to -483bp] in Chapter II). In Figure 2, the frequency of methylation in each site within this putative core region is represented for both DNA samples, together with the indication of the *cis*-elements identified.

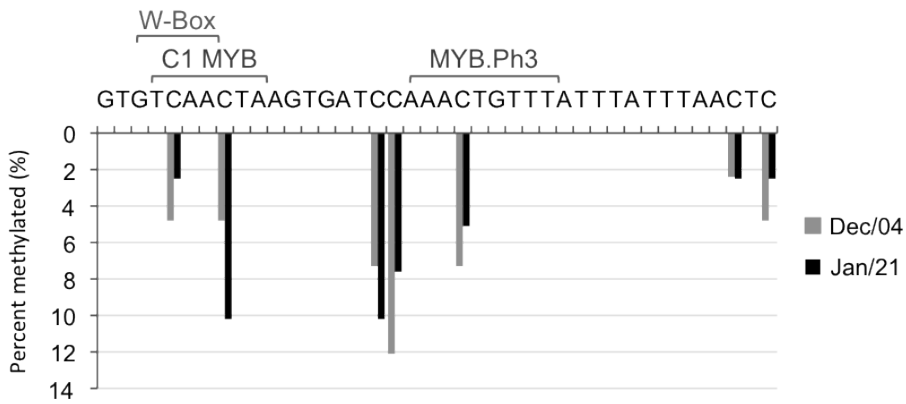

Figure 2. *PrdCBF2* promoter region showing the highest cytosine methylation frequencies in flower bud DNA samples from Dec/04 and Jan/21. The position of putative *cis*-elements predicted for this region are shown.

3.2 Differential expression of PrdCBF2 in floral organs

To elucidate the expression pattern of *PrdCBF2* within floral whorls, an expression study was conducted in cDNA pools collected from calyx and

inner floral whorls (corolla, stamens and carpels). Expression of *PrdCBF1* and *PrdDHN1* was also analyzed, in order to gain additional insights on the expression of cold-regulated genes in these whorls (Figure 3). The time points analyzed corresponded to late January collections, when blooming started to occur and expression of *PrdCBF1, -2* and *PrdDHN1* was reduced (Figures 4 and 5 in Chapter IV). Therefore, the expression of these genes in separated whorls from fully opened flowers was also low and faintly detected in some cases. However, and in the particular case of *PrdCBF2* and *PrdDHN1* transcript levels were somewhat higher in calyx cDNA than in cDNA from inner floral whorls. These results suggest that expression of these genes may be more abundant in sepals than in petals and reproductive organs.

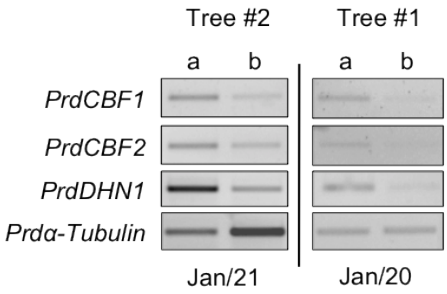

Figure 3. Analysis of the *PrdCBF1*, *PrdCBF2* and *PrdDHN1* transcript level in calyx (a) and inner floral whorls (b), obtained from late-January flower buds. Tree #2 was analyzed in year-I (Jan/21); Tree #1 was analyzed in year-II (Jan/20).

4. Discussion

DNA methylation has been shown to play a determinant role in regulating gene expression in a tissue-specific or developmental stage-dependent manner throughout plant development (Chan et al., 2005; Zhang et al., 2010). In the present chapter, we determined the DNA methylation pattern of a specific promoter region of *PrdCBF2* in DNA from flower buds collected at two different developmental stages. No significant differences

were found between the methylation patterns for each developmental stage. In both samples, the global DNA methylation level was low and the methylated cytosines were randomly distributed in a great extent of the clones. These results seem to indicate that the differences in *PrdCBF2* gene expression, between fall and winter stages may not be correlated to a specific methylation pattern, at least in what concerns the targeted promoter region. Nevertheless, in a specific set of clones obtained from both DNA pools, it was possible to detect a region where methylation cytosines showed to be enriched, particularly in CHH contexts.

RdDM is the main mechanism related to perpetuation of CHH asymmetric methylation, given the lack of template-based maintenance as observed in symmetric methylation (Teixeira and Colot, 2010). siRNAs mediate *de novo* methylation in many genomic regions through RdDM. A functional model has been proposed in which long double stranded RNAs are produced from DNA tandem repeats or TEs (mainly from heterochromatic origin) and processed by the nuclear RNA interference (RNAi) machinery, forming siRNA molecules of 24 nucleotides long (Matzke et al., 2009; Teixeira and Colot, 2010). Single strand siRNAs then associate to a silencing protein-complex, which may bind to complementary DNA regions. DNA methylation is then induced by DRM2 MTase, which is recruited by that silencing complex (Matzke et al., 2009; Teixeira and Colot, 2010). Therefore, it would be interesting to investigate if the methylation-target region identified in *PrdCBF2* promoter region could be a putative target-site for complementary siRNA molecules.

Sequenced clones showing this putative methylation target-site were obtained in two independent DNA pools but the overall methylation frequencies in this region did not reach values higher than 10%. Considering that site-specific promoter methylation has been associated with tissue-specific gene expression (Zhang et al., 2006), we hypothesized that this specific pattern of cytosine methylation in *PrdCBF2* promoter could

be associated with a specific tissue(s) within the flower bud. This prompted us to investigate if *PrdCBF2* could be differentially regulated in floral organs. Gene expression analysis on cDNA pools from calyx and inner floral whorls revealed that *PrdCBF2* and *PrdDHN1* transcripts were slightly more abundant in the former. These results are in agreement with the expression pattern determined for *CBF* genes in *Arabidopsis* (Novillo et al., 2007). In transgenic plants expressing *AtCBF1-3* promoter::*GUS* fusions, GUS activity in floral organs after 3h exposure to 4ºC, was only detected in sepals and mature siliques. Additionally, the promoter of *AtCBF2* was also active in sepals under controlled conditions (Novillo et al., 2007). Although the role of *CBF* genes in flower development is still unknown these expression results point out for a differential role of these TFs during floral organ development. An intriguing fact, although still requiring further confirmation in additional transgenic lines, is that PrdCBF2 overexpression in *Arabidopsis* caused an impairment of flower development (Supplementary Figure 2 in Chapter III), hampering anthesis.

Further analyzes should be performed in order to validate and associate the predicted methylation-target region on the *PrdCBF2* promoter to individual organs or tissues. Such analyses, together with detailed tissue-specific gene expression data could confirm the regulatory role of this region, where putative *cis*-elements related to different regulatory pathways were already indentified. Particularly, two MYB-related recognition sites (MYB-c, Figure 4b in Chapter II) were found, which are putative binding-sites for R2R3 MYB TFs related to the anthocyanin biosynthetic pathway: C1 in maize aleurone (Bodeau and Walbot, 1992) and MYB.Ph3 in epidermal cells from petals in petunia (Solano et al., 1995). In addition to the role in organ pigmentation (Tanaka et al., 2008) and leaf senescence (Hoch et al., 2001), anthocyanins have been also implicated in cold acclimation, probably related to their role in photo-oxidation protection (Hannah et al., 2005). Ectopic expression of a peach CBF in apple lead to an increased

accumulation of anthocyanins in cold acclimated leaves (Wisniewski et al., 2011). However, leaves from transgenic potato overexpressing an *Arabidopsis* CBF were found to contain lower anthocyanin levels as compared to wild type (Pino et al., 2008). Although this may reflect species-specific strategies for stress response, it may also reflect differences in the corresponding CBF regulons (Wisniewski et al., 2011). Thus, it would be also interesting to study the effect of PrdCBF2 in anthocyanin accumulation, and how would it affect specific floral organ development.

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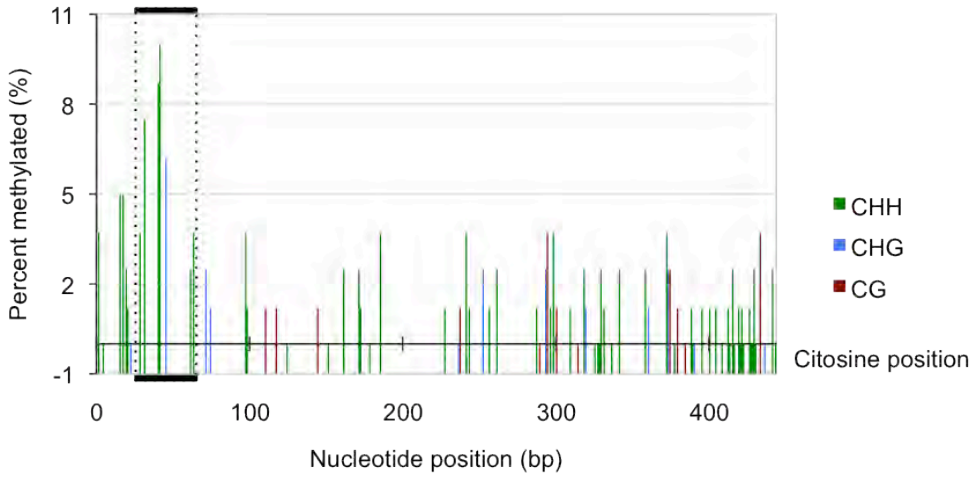
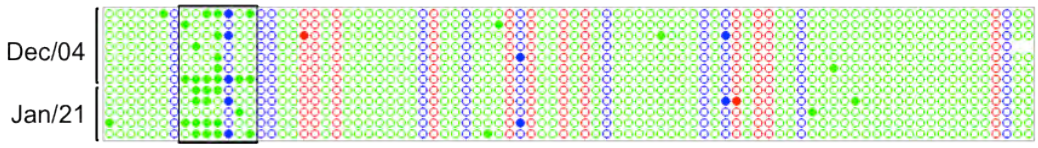
7. Supplementary Data



Supplementary Figure 1. Methylation frequencies at each cytosine position within *PrdCBF2* promoter fragment targeted for bisulfite sequencing, in DNA samples obtained from almond flower buds. The sequences obtained after bisulfite sequencing for both developmental stages (Dec/04 and Jan/21) were analyzed as a unique pool. Each cytosine is represented by the correspondent sequence context: CHH (green), CHG (blue) and CG (red). The black dotted box highlight a putative methylation-target region due to the highest frequencies observed.



Supplementary Figure 2. Global methylation pattern observed in individual sequences showing methylation within the predicted target region (black box) highlighted in Figure I. Each cytosine within *PrdCBF2* promoter fragment is represented by a circle according to the sequence context: CHH (green), CHG (blue) and CG (red). Open circles represent unmethylated cytosines.

CHAPTER VI

Final Conclusions and Future Perspectives

Almond shows a high genetic variability, being therefore considered a unique source of interesting traits that can be introgressed in other Prunoideae, more particularly, in species with less variability, such as peach. However, prior to exploring natural genetic variability, it is crucial to identify and characterize the genetic mechanisms controlling key traits for crop improvement.

Given its impact on fruit production, the role of temperature in dormancy break and blooming time in temperate fruit trees is well documented. Low temperatures are assumed to play a dual role in dormancy regulation, as they promote endodormancy break (according to specific chilling requirements) but later avoid ecodormancy break. This regulation often leads to changes in flowering time in different years. As a consequence of global warming, the impacts of inadequate chilling exposure (during endodormancy) or early exposure to mild temperatures (during ecodormancy) also need to be considered, according to the chilling requirements of a given genotype grown in a given climatic region. Thus, a main research topic in these species has been the genetic and molecular mechanisms involved in temperature requirements for dormancy break.

The present thesis reported the isolation and functional characterization of two genes (*PrdCBF1* and *PrdCBF2*) involved in low temperature signalling in almond. As members of the highly conserved *CBF* gene family of transcription factors, these genes showed common sequence features with *CBF*s from other plants, in both transcribed and promoter regions. *PrdCBF1* and *PrdCBF2* genes are induced during low temperature (below 12ºC) exposure and cold acclimation, being only transiently activated by other stress signals such as dehydration and ABA. Gene expression studies performed using *in vitro* propagated plants also revealed that transcription of both *PrdCBF*s might be under light and/or circadian regulation. In fact, *PrdCBF1* and -2 transcription during cold exposure showed to be favoured towards the end of day. Interestingly, the expression

pattern of *PrdDHN1* under control growth conditions followed a light/circadian-like pattern of gene expression, increasing towards the night and decreasing afterwards. These results suggested that cold-responsive signalling pathways in this species could be positively regulated close to dusk. If true, this regulation could act as a signalling mechanism related to thermoperiod transitions, which are pointed out as an important aspect in seasonal dormancy. In this way, it would be interesting to determine the pattern of *PrdCBF1* or *PrdCBF2* gene expression during normal growth conditions and compare it to that described for *PrdDHN1*. As referred in Chapter II, *CBF* genes may follow a circadian-like induction pattern under control growth conditions, although never reaching expression levels compared to those observed during cold response. Therefore, the use of more accurate transcript quantification methods, such as real-time PCR, would be preferable.

Genomic analysis by Southern blot indicated that the *PrdCBF* family was composed of at least four genes. Further nucleotide similarity search (*in silico*) in the peach genome sequence suggested at least another member of the family, and predicted that these genes could be located in close proximity. In agreement, bin mapping analysis on the *Prunus* reference map, associated *PrdCBF1* and *PrdCBF2* to the same genomic region, within linkage group (LG) 5. In turn, *PrdDHN1* was mapped onto LG7. The corresponding chromosome regions associated with *PrdCBF*s and *PrdDHN1* overlapped with previously identified QTLs related to blooming time. However, since this mapping approach only provides a rough association to a specific chromosome fragment, possible associations with previously mapped genes, markers or QTLs have limited significance. Therefore, further association studies and fine mapping of these genes may help to clarify this association.

The transactivation activity of PrdCBF1 and PrdCBF2 was evaluated by heterologous expression in *Arabidopsis*. While both genes

showed to be functional during transient expression in protoplasts, similar conclusions could not be drawn *in planta*. Besides the low transformation efficiency, the constitutive expression of these genes, driven by the 35S promoter, lead to the generation of dwarf transgenic lines with delayed development and no (or poor) viable seed set. Although the use of a strong constitutive promoter could be questioned as a possible explanation of such traits, the 35S promoter has been a classical choice in the majority of the publications related to the functional characterization of *CBF* genes in *Arabidopsis*. Nevertheless, in one transgenic line, overexpression of PrdCBF2 lead to the induction under control growth conditions of several endogenous cold-regulated genes, and also to an increase in freezing tolerance, as compared to wild-type plants.

Although *CBF* genes have been widely studied, their role during cold acclimation had never been addressed under natural conditions. To follow the gene expression pattern of *PrdCBF1* and *PrdCBF2* under these conditions, we selected a group of adult almond trees growing in close proximity and collected flower buds and shoot samples at periodic intervals. The analyses conducted in two years generated relevant information regarding the function of both *PrdCBF*s and also of additional genes involved in cold acclimation and in flower bud development along the dormancy period. In fact, *PrdCBF1* and *PrdCBF2* may play different roles during seasonal development. *PrdCBF2* transcripts were consistently more abundant from mid-fall to early winter, when temperatures were close to chilling levels. Thus, we suggest that *PrdCBF2* is an active integrator of the cold acclimation pathway, which is induced during fall after dormancy induction. In turn, *PrdCBF1* expression was not consistent in both years, being detected since late summer in shoot samples.

During ecodormancy, cold has a negative effect on growth and may help maintaining the full-acclimated state. However, the occurrence of warm temperatures induces a promotive effect over growth, probably also

following specific temperature requirements. A striking result in our study was the global decline in the expression of *PrdCBF1*, *PrdCBF2* and their putative target *PrdDHN1* prior to bud break in mid-winter, in both shoots and flower buds. We hypothesized that this decline could be a cause or a consequence of a deacclimation process expected to occur during growth resumption. Thus, the cold signalling pathways mediated by both *PrdCBFs* (or at least *PrdCBF2*), which probably include *PrdDHN1* and other cold-regulated genes, may control, or be dependent on, the plants' deacclimation resistance. To study this association in more detail it would be interesting to identify specific factors that may negatively regulate *PrdCBF1* and *2*, or *PrdDHN1* itself, during deacclimation. Although the DNA methylation state of a specific *PrdCBF2* promoter region did not show any variation within different developmental stages, epigenetic mechanisms are still valuable candidate players in this regulation. Besides, it would be interesting to further investigate if deacclimation resistance also varies among cultivars with different chilling requirements.

DORMANCY ASSOCIATED MADS-box (DAM) genes are candidate members of the CBF regulon. Considering the proposed involvement of these genes in the control of growth and development during dormancy, it would be interesting to investigate if *PrdCBF1* or *-2* could specifically regulate *DAM* homolog genes in almond. In peach, the level of expression of several *DAM* was correlated to the level of chilling necessary to break dormancy. In this scenario it is possible that CBFs could play a role in determining variety-specific chilling requirements.

The present work elucidated for the first time in a fruit tree species the role of *CBF*-mediated low temperature signalling pathways during ecodormancy break. In addition, it also provided valuable information regarding other candidate genes involved in flower bud development (*PrdGA20OX*, *PrdGA2OX*, *PrdMADS1* and *PrdMADS3*). Although the specific function of these genes stands only based on homology to other

known genes from model plants, the consistent gene expression patterns obtained for dormancy-activity transitions is an evidence of a specific function. In the future, after functional validation, it would be important to investigate if these particular genes are also regulated by temperature or other environmental factor.

Since molecular research in fruit trees is usually limited by the long life cycle/juvenile period, among other factors, valuable approaches for gene functional analysis, such as reverse genetics, may be difficult to perform. Therefore, comparative genomic and transcriptomic studies in cultivars having different temperature requirements could be relevant to further characterize the genes studied in the present work. These approaches may validate their specific role during flower bud development, in addition to explore the natural variability occurring within this species. Considering the high genetic sinteny occurring among the *Prunus* genus, this data could be easily transferred to other species, providing important tools to improve traditional breeding and to establish more profitable orchards.

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